DOCTOR OF PHILOSOPHY

The effects of intra-dorsal raphe administration of strychnine on rat behaviour and its interactions with ethanol

Greig, Scott James

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by

Scott James Greig

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Abbreviations

2-AG	-	2-arachidonyl-glycerol
5-HIAA	–	5-hydroxyindoleacetic Acid
5-HT	-	5-hydroxytryptamine, Serotonin
5-HTP	-	5-hydroxytryptophan
5,7-DHT	-	5,7-dihydroxytryptamine
5,6-DHT	-	5,6-dihydroxytryptamine
8-OH-DPAT	-	8-hydroxy-2-(di-n-propylamino)tetralin
aCSF	-	Artificial Cerebrospinal Fluid
ACTH	-	Adrenal Corticotrophic Hormone
ADH	-	Alcohol Dehydrogenase
AEA	-	Anandamide
ALDH	-	Aldehyde Dehydrogenase
AMPA	-	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
Ca^{2+}	-	Calcium
Cl^{-}	-	Chloride
CNS	-	Central Nervous System
CRF	-	Corticosterone Releasing Factor
DMN	-	Dorsomedial Nucleus

DRN	-	Dorsal Raphe Nucleus
DSM	-	Diagnostic and Statistical Manual of Mental Disorders
GABA	-	γ-aminobutyric Acid
GlyT	-	Glycine Transporter
GR	-	Glucocorticoid Receptor
HPA	-	Hypothalamic Pituitary Adrenocortical
ICD	-	International Classification of Diseases
i.p.	-	Intraperitoneal
KCC2	-	K^+-Cl^- co-transporter
KCl	-	Potassium Chloride
mCPP	-	m-chlorophenylpiperazine
mIPSCs	-	miniature inhibitory post-synaptic currents
MR	-	Mineralocorticoid Receptor
MRN	-	Median Raphe Nucleus
nACh	-	Nicotinic Acetylcholine Receptor
NaCl	-	Sodium Chloride
NKCC1	-	Na^+-K^+-$2Cl^-$ co-transporter
NMDA	-	N-methyl-D-aspartic acid
PAG	-	Periaqueductal Grey

PCPA	-	para-chlorophenylalanine
PVN	-	Paraventricular Nucleus
s.c.	-	Subcutaneous
s.e.m.	-	Standard Error of the Mean
SSRI	-	Serotonin Selective Reuptake Inhibitors
TauT	-	Taurine Transporter
TM	-	Transmembrane Domain
VGLUT3	-	Vesicular Glutamate Transporter-3
vlPAG	-	Ventrolateral Periaqueductal Grey
vmPFC	-	Ventromedial Prefrontal Cortex
VTA	-	Ventral Tegmental Area

Units of Measurement

M	-	Molar
mM	-	millimolar
m	-	Meter
cm	-	Centimetre
mm	-	Millimetre

ga	-	Gauge
ml	-	Millilitre
µl	-	Microlitre
nl	-	Nanolitre
hr	-	Hours
min	-	Minutes
g	-	Grams
kg	-	Kilograms
µg	-	Micrograms
ng	-	Nanograms
pg	-	Picograms

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Declaration

I hereby certify that I, Scott Greig, am the author of this thesis, that all references in this manuscript have been consulted by me, that this thesis is a record of work carried out by me, and that it has not been previously accepted for a higher degree.

Signature of Candidate Date

(Scott James Greig)

I hereby certify that the candidate has fulfilled the conditions of the Ordinance and Regulations for the Doctor of Philosophy in the University of Dundee.

Signature of Supervisor Date

(Professor Jeremy Lambert)

Signature of Supervisor Date

(Dr Delia Belelli)

Signature of Supervisor Date

(Professor David JK Balfour)

Summary

The study of the neurobiological mechanisms which underpin the generation of anxiety has garnered significant attention due to the prevalence of anxiety disorders and the distress these pathologies can have on the individual, their family and society in general. However, the term ‘anxiety’ is not unitary but encompasses a diverse range of affective, cognitive, endocrine and behavioural processes. Previous studies have identified a population of inhibitory strychnine-sensitive glycine receptors, which are expressed on the serotonergic neurons which originate from the dorsal raphe nucleus. In addition, the serotonergic neurons of the dorsal raphe nucleus, which mediate the release of the neurotransmitter serotonin, have been implicated as a component of the neurocircuitry which underpins the generation of an anxious state. Therefore, the receptor antagonist strychnine was administered into the dorsal raphe nucleus of Sprague-Dawley rats and behaviour was investigated in the open field, elevated plus-maze and locomotor activity box.

The intracerebral microinjection of strychnine into the dorsal raphe nucleus had no effect on anxiety-like behaviours or locomotor activity in animals exposed to the open field. The elevated plus-maze methodology was validated, prior to the investigation of the strychnine-sensitive glycine receptors, by replicating the previously reported anxiolytic effects in response to the systemic administration of chlordiazepoxide. Animals were divided into two groups and either remained in the home cage or were repeatedly exposed (1 hr/daily) to an elevated open platform stressor over 10 days prior to testing in the elevated plus-maze. In the elevated plus-maze, the administration of strychnine into the dorsal raphe nucleus selectively reversed the increase in the percentage of the total time spent exploring the open runways of animals pre-exposed to the elevated open

platform stressor. However, there was no effect of strychnine on the anxiety-like behaviours of animals which remained in the home cage prior to testing in the elevated plus-maze. In addition, the administration of strychnine into the dorsal raphe nucleus did not significantly influence the locomotor activity in either group. However, the administration of the endogenous agonist glycine into the dorsal raphe nucleus did not appear to elicit an anxiolytic-like effect. Therefore, the data suggest that the strychnine-sensitive glycine receptors of the dorsal raphe nucleus may play a role in the habituation to an inescapable stressor.

Furthermore, previous studies have identified that the strychnine-sensitive glycine receptors expressed in the dorsal raphe nucleus are potentiated by low, physiologically relevant, concentrations of ethanol. Therefore, it was hypothesised that a component of the anxiolytic properties of ethanol may be mediated by the strychnine-sensitive glycine receptors of the dorsal raphe nucleus. However in response to the systemic administration of ethanol, the anxiolytic properties of ethanol in the elevated plus-maze appeared to be masked by the sedative component of the drug. Therefore, an alternative route of administering ethanol was investigated. The intracerebroventricular administration of ethanol appeared to elicit an anxiolytic-like effect, as measured by the elevated plus-maze, in the absence of an overt change in locomotor activity. Therefore, the effect of concurrent intracerebral administration of strychnine into the dorsal raphe nucleus and ethanol into the lateral ventricle was investigated in the elevated plus-maze and a locomotor activity box. Contrary to the original hypothesis, the administration of strychnine into the dorsal raphe nucleus did not reverse a component of the anxiolytic effects of the intracerebroventricular administration of ethanol in the elevated plus-maze. However, contrary to the previous studies in the elevated plus-

maze, the administration of strychnine into the dorsal raphe nucleus reduced the locomotor activity of the animals administered the artificial cerebrospinal fluid vehicle, but not ethanol, into the lateral ventricle. In addition, the administration of strychnine into the dorsal raphe nucleus reduced the duration of the trial time spent exploring the central zone of the locomotor activity box suggesting an anxiogenic-like effect. In addition, the administration of ethanol into the dorsal raphe nucleus was investigated in the elevated plus-maze. However, the administration of ethanol into the dorsal raphe nucleus did not significantly influence either the indices of anxiety-like behaviours or the locomotor activity in the elevated plus-maze. However, this may have been due to the diffusion of ethanol from the target site.

Collectively, the results of the present study suggest that the administration of strychnine into the dorsal raphe nucleus mediates the suppression of locomotor activity and an anxiogenic-like effect. However, these effects appear to depend upon the basal anxiety state of the animal. At present, the data do not support the hypothesis of an interaction between the strychnine-sensitive glycine receptors of the dorsal raphe nucleus and the anxiolytic properties of ethanol. However, potentially confounding variables associated with the methodology may have masked measurable behavioural in response to the administration of ethanol. Therefore, the interpretation of the results must be considered in the context of these limitations. However, additional neurochemical and physiological measures could be recruited to the study to further elucidate the role of the strychnine-sensitive glycine receptors localised within the dorsal raphe nucleus.

The aims of the present study

The principal aim of the project was to investigate the role of strychnine-sensitive glycine receptors in the DRN of the rat. Its specific objectives were to:

a. test the hypothesis that stimulation of the receptors elicited anxiolytic-like behaviour;
b. explore the potential interaction between the receptors and the anxiolytic properties of ethanol.

1. Introduction

1.1. Anxiety

The term ‘anxiety’ refers to an emotional state generated in response to future events which have an ambiguous outcome. Anxiety can become pathological; however, it is also an important evolutionary adaptation which serves to protect healthy individuals from harm in potentially threatening situations. Pathological anxiety is a common condition and has received extensive investigation due to the distress caused to the individual, their family and society in general. Although psychological therapies have been beneficial, in the majority of cases pharmacological interventions are the most common and efficacious treatments (Sandford *et al*, 2000). The expression of anxiety involves three critical components - the evaluation of the threat, the subjective experience of the situation and the expression of an anxious state (LeDoux, 2003). Therefore anxiety is the manifestation of complex cognitive, affective and behavioural processes. Under non-pathological conditions, the expression of anxiety has evolved to suppress responses which may increase the likelihood of disadvantageous outcomes. Classically, this was considered to consist of flight, fight or freezing behaviours (Cannon, 1929) but has been expanded to include approach-avoidance, anticipatory fear coupled with an increase in arousal (Yerkes, 1921). The study of anxiety overlaps both neurobiological and psychological disciplines and therefore what constitutes the term ‘anxiety’ continues to evolve. Physiological measures such as increased arterial blood pressure, tachycardia and excessive secretion of glucocorticoids are informative but are not definitive of anxiety *per se* (Millan, 2003). However, the study of individuals diagnosed with ‘anxiety disorders’ and the investigation of the diverse pharmacological agents which are effective in their treatment has

implicated key components in the neuronal mechanisms which underpin the expression of anxiety.

1.1.1. Anxiety disorders

Anxiety is a normal emotional state in healthy individuals. However, when it interferes with the normal day to day functioning of an individual it can be severely debilitating. It can be diagnosed as a symptom of a wider psychiatric disorder or the primary symptom in a group of distinct illnesses, which share some similar characteristics, collectively referred to as the 'anxiety disorders' (Sandford *et al*, 2000). The expression of anxiety is associated with an increase in physiological measures of arousal in healthy individuals. However, the clinical diagnosis of distinct 'anxiety disorders' would suggest that pathological anxiety is not simply a maladaptive state of arousal. Therefore, the term anxiety is not a unitary term by reflects a diverse array of neurological processes. This diversity was reflected in the two systems of categorical classification of the anxiety disorders – the ICD-10 (World Health Organisation, 1992) and DSM-IV systems (American Psychiatric Association, 1992). Both systems share commonalities and include panic disorder, generalised anxiety disorder, agoraphobia, specific phobia, social phobia, obsessive compulsive disorder, acute stress disorder and post-traumatic stress disorder. In 2013, the publication of the DSM-5 system introduced a number of changes to the DSM-IV system of characterising the anxiety disorders. These changes included the dissociation of obsessive compulsive disorder, post-traumatic stress disorder and acute stress disorder into distinct chapters. However, the sequential order of these chapters reflects the close relationships between them (http://www.dsm5.org/Documents/changes%20from%20dsm-iv-tr%20to%20dsm-5.pdf; accessed 10/10/13).

1.1.2. The distinction between state and trait anxiety

It is important to distinguish between the ‘the anxious state’ and ‘trait anxiety’. The ‘anxious state’ refers to the expression of an anxious response at a particular time in response to a specific event which typically becomes diminished following its resolution. Therefore not only does the individual’s response change over time, but the subjective experience of the aversive event changes over time. However, ‘trait’ anxiety refers to the persistent manifestation of high anxiety which is characteristic of the individual and influences both environmental and social interactions. In animals, the behavioural tests of anxiety-like behaviours have been used to induce an ‘anxious state’ by the presentation of a punished response or a novel environment. However, the modelling of ‘trait’ anxiety in animal models is more difficult.

1.1.3. The peripheral versus central origins of anxiety

Historically, emotional states were thought to be derived from the peripheral and not the central nervous system. James and Lange proposed that the expression of emotion was evoked by the feedback of peripheral sensory receptors (reviewed by Pratt, 1992). Therefore the expression of emotional states was initiated by physiological changes in the periphery. However, this view was challenged by several experimental findings. Cannon (1927) reported that the transaction of the spinal cord at the cervical level in dogs did not abolish normal rage reactions despite the severing of sensory receptor feedback. Later experiments investigated the peripheral administration of adrenaline, which does not cross the blood-brain barrier, in healthy volunteers subjected to psychological cues (Schachter and Singer, 1962). The subjective experience of the individuals was predominantly dependent on the psychological cues as

opposed to the administration of adrenaline suggesting that the generation of emotion is central in origin. However, the activation of the sympathetic nervous system mediates the expression of the physiological symptoms of anxiety in healthy individuals. These include tachycardia, sweating, flushing and shaking in addition to motor tension (Davies *et al*, 2010). One exception has been reported in patients diagnosed with panic disorder. These individuals commonly misinterpret innocuous physical symptoms as sign of illness or an impending panic attack (Clark, 1986). In a process referred to as 'catastrophic cognitive misinterpretation', this misinterpretation of physical symptoms generates anxiety in the individual which activates the sympathetic nervous system. The activation of the sympathetic nervous system exacerbates the physical symptoms which were initially misinterpreted, generating further anxiety, and this cycle ultimately induces a panic attack.

1.1.4. The investigation of anxiety in animals

Our understanding of anxiety is predominantly derived from physiological and behavioural studies in animals in addition to functional imaging studies in humans. Functional imaging studies have provided insight into the mechanisms of pathological anxiety and the more subjective aspects of normal anxiety. However, animal studies have proven to be a more useful tool in the investigation of the expression and evaluation components of anxiety (Pratt, 1992). In such studies, behavioural measures such as punished responding, approach-avoidance and freezing behaviours have been assumed to reflect the emotional state of the animal (Millan, 2003). The behavioural tests of anxiety are broadly categorised into two groups – those that evoke an anxious state through the presentation of a conditioned stressor and those that generate an anxious state through the presentation of an unconditioned stressor.

Conflict tasks, such as the Geller-Seifter, Skinner and Vogel tasks, present a reward (i.e. food or water) in the absence and presence of a punishing stimulus (i.e. shock). Geller *et al*. (1960) reported that rats were trained to press a lever for a food reward in the presence of a visible cue. However, in subsequent trials the lever still presented the food reward but was paired with a concurrent electric shock to the feet. The rats showed a marked suppression of lever pressing during the punished trials. Previously, a similar task had been developed by Estes and Skinner (1941). In response to a lever press, a food reward is presented and after a brief delay an acoustic tone sounds, which is followed by a foot shock. Response inhibition occurs after the animal has received several pairs of tone and shock and an association between the two forms. Upon the onset of the tone, responses of the animal for the food reward are decreased until cessation of the tone itself.

Anxiolytic drugs have been found to reproducibly increase punished responding for the reward. Therefore, punished responding is considered a measure of anxiety-like behaviours. Although these conflict tasks have been found to be reliable and reproducible in the investigation of a number of anxiolytic drugs, they require training schedules and typically rely upon food restriction or water deprivation to induce the motivational drive to respond for the reward.

More ethological tests of anxiety have utilised the innate fear of novel environments as the unconditioned stressor to generate anxiety. These tests include the elevated plus-maze, the open field, the social interaction test and the light-dark box (Pellow *et al*, 1985; Hall, 1934; File and Pope, 1974; Lorenzini *et al*, 1984). As the animals do not require training, these tasks have become increasingly popular in behavioural studies due to the simplicity and short duration of the experimental protocol. However, there is a relative lack of

reproducibility and reliability in such tasks due to the array of methodological differences in the literature (e.g. dimensions, light levels, handling etc.).

The results of behavioural tests in animal studies must therefore be considered in the context of the test and the nature of the stressor used (e.g. conditioned versus unconditioned) to induce an anxious state. In part, this may account for differences in the effects (i.e. anxiolytic, anxiogenic or no effect) of experimental treatments tested in a battery of such tasks. In addition, the diverse nature of the tests and the stimuli used to elicit the expression of anxiety reflects the diversity of defensive behaviours which are sensitive to pharmacological manipulation.

1.1.5. Hierarchy of defensive behaviours

Using an ethological approach, Blanchard *et al*. (2011) investigated factors which determine the expression of specific defensive behaviours such as avoidance, freezing, escape and attack. The authors reported that in the absence of an escape route, wild rats exposed to a human stressor elicited freezing behaviours when the proximity was greater than 1 m. However, defensive attack was observed when the proximity was reduced to less than 0.5 m (Blanchard and Blanchard, 2011). Conversely, the addition of an escape route elicited flight as opposed to freezing behaviours (Blanchard and Blanchard, 1989). Therefore, the proximity of the threat was a reliable determinant of the defensive strategy adopted (Blanchard *et al*, 2011). In addition, Blanchard and Blanchard (1989) reported differences in the defensive strategy adopted by animals presented with a predator stressor and those presented with the odour of the predator. Exposure of rats to a cat odor block increased risk assessment as measured by an increased incidence of stretch-

attend movements, freezing and avoidance behaviours (Blanchard *et al*, 1990b; Blanchard *et al*, 2001).

The authors coined the term ‘defensive distance’ (i.e. the proximity of the threat) as a critical determinant of the defensive strategy adopted by the animal in response to threatening stimuli. The presence of a proximal threat elicits either flight or fight responses depending on environmental factors (i.e. the potential for escape or hiding), which are considered fear mediated responses. However, the presence of a distal or potential threat initiates risk assessment behaviours which are thought to reflect anxiety-like processes (Blanchard *et al*, 2011). The authors reported that the initiation of risk assessment behaviours and the evaluation of the threat were a critical determinant of the defensive strategy adopted (Blanchard *et al*, 2011).

McNaughton and Corr (2004) suggested that the appropriate defensive behaviour elicited may be mediated by distinct neuronal systems within the circuitry of anxiety. This hierarchy of defensive behaviours suggests that more primitive defensive responses such as fight/flight are mediated by lower level nuclei in the hierarchy, such as the periaquductal grey (PAG) and locus coeruleus, in response to immanent proximal threats. An intermediate level, comprising the amygdala and septo-hippocampal system, mediates defensive strategies in response to distal threats. Whereas higher centres, such as the prefrontal cortex, mediate the defensive behaviours in response to perceived or potential threats which may require more complex cognitive responses. In this arrangement, the higher levels are able to feedback down to inhibit the responses of the lower levels in the hierarchy (*figure 1.1.*; McNaughton and Corr, 2004).

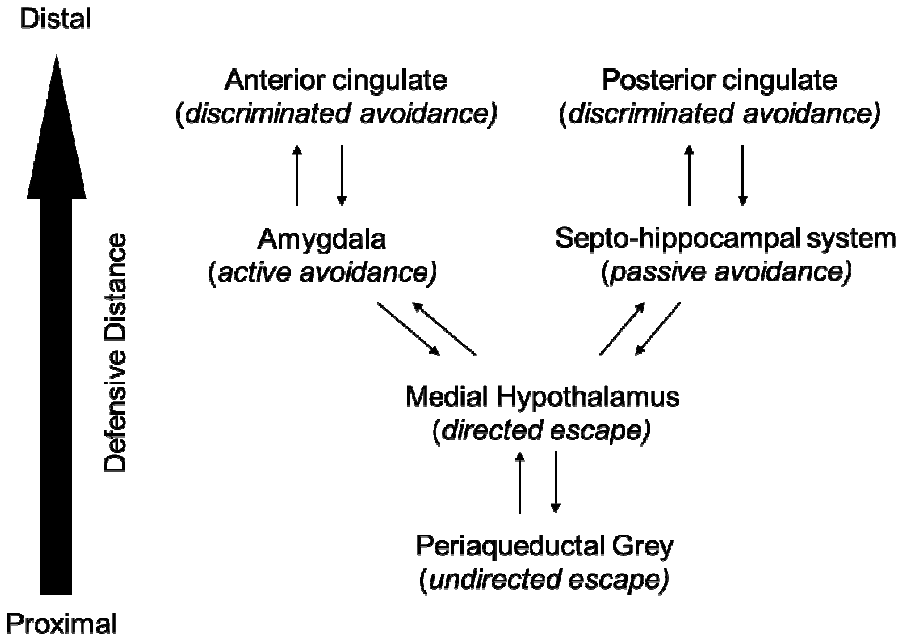

Figure 1.1. – The hierarchical organisation of the neuroanatomical substrates of defensive behaviours. The hierarchy of defensive behaviours is derived from the work of Blanchard and Blanchard (2011) who reported that the defensive response of an animal was influenced by the proximity of the threat. Therefore, McNaughton and Corr (2004) hypothesised that the neuroanatomical substrates which mediate these defensive responses may be organised in a similar hierarchical arrangement. Threats such as a proximal predator or a dominant conspecific induce flight/fight responses mediated by the PAG. However, distal or perceived threats are mediated by higher cognitive centres as the defensive response requires more complex defensive strategies. Adapted from Gray and McNaughton (2003).

1.2. Neurocircuitry of anxiety

The neuroanatomy of anxiety is historically associated with the Papez circuit of emotion, which includes the hypothalamus, anterior thalamus, cingulate gyrus and hippocampus (Papez, 1937). Papez (1937) proposed that sensory information from the periphery is projected to the mammillary body of the hypothalamus via the thalamus. This is then relayed to cortical structures via the cingulate gyrus which is considered the site of the subjective emotional experience. Projections back to the hypothalamus, via the hippocampus, mediate the downstream expression of emotion (Papez, 1937).

More recent studies have implicated a number of anatomical substrates omitted in the original Papez circuit which play a role in the mediation of anxiety. Systematic analysis of the connectivity of these nuclei has led to the investigation of distinct neuronal systems which mediate specific responses to aversive stimuli. These include the 'defensive system' which mediates the immediate responses to proximal threats and is analogous to panic attacks (Sandford *et al*, 2000). Another is the 'behavioural inhibition system' which was originally proposed by Gray (reviewed by Gray and McNaughton, 2003). This system is thought to mediate the suppression of behaviours which increase the likelihood of adverse events and this may include the inhibition of behaviours mediated by the defensive system (Sandford *et al*, 2000). These two systems have been the most extensively investigated and are thought to work in concert with one another but others are likely to play a role. In the present review, specific anatomical substrates of the circuitry are discussed in brief with an emphasis on the role of the neurotransmitter serotonin at such centres when appropriate.

1.2.1. Hypothalamic Nuclei

The role of the hypothalamus in the mediation of anxiety is twofold. Firstly, the hypothalamus is an important component in the initiation of both the cardiovascular and endocrine responses to stressful stimuli due to the role of the paraventricular nucleus (PVN) in the hypothalamic-pituitary-adrenal (HPA) axis. The activation of the HPA axis is considered a hallmark of the stress response (DiMicco *et al*, 2002). Stress-induced increases in glucocorticoids are evoked by the release of adrenal corticotrophic hormone (ACTH), which is triggered by the secretion of corticotrophin releasing factor (CRF) into the

hypothalamic portal system at the median eminence (DiMicco *et al*, 2002). The neurons of the PVN synthesise CRF (Jankord and Herman, 2008). In addition, the projections from the PVN to the spinal cord are thought to mediate the cardiovascular responses to stress. Microinjection of the $GABA_A$ receptor antagonist bicuculline, the excitatory kainic acid and NMDA into the PVN have been reported to increase heart rate and blood pressure mimicking responses to stress (Haywood *et al*, 2001; Schlenker *et al*, 2001; DiMicco *et al*, 2002).

In addition, the hypothalamic nuclei have been implicated in the mediation of the behavioural responses to anxiogenic stimuli. Disinhibition of the dorsomedial nucleus (DMN), induced by the central administration of the $GABA_A$ receptor antagonist bicuculline, evokes tachycardia, increased secretion of ACTH, and an anxiogenic-like phenotype in the social interaction test and elevated plus-maze (Shekhar, 1993; Shekhar *et al*, 1993; Shekhar and Katner, 1995). Conversely, the intra-DMN administration of the $GABA_A$ receptor agonist muscimol induced an anxiolytic-like effect in both tests (Shekhar, 1993; Shekhar and Katner, 1995). In addition, the administration of chlordiazepoxide, diazepam and midazolam into the mammillary body, which is a component of the original Papez circuit, were reported to increase punished responding in a Geller-Seifter conflict task (reviewed by Menard and Treit, 1999). The contribution of the individual hypothalamic nuclei to the mediation of defensive behaviours remains to be determined and may be mediated, in part, by distinct circuits within the hypothalamus itself. Canteras *et al*. (2012) proposed a predator-responsive medial hypothalamic circuit and a distinct social/reproductive medial hypothalamic circuit based on c-fos immunoreactivity in response to predator associated cues.

1.2.2. Amygdala

The amygdala is extensively interconnected with the cortex, locus coeruleus, midbrain, striatum and brain stem (Sandford *et al*, 2000) and plays a role in the mediation of the locomotor, neuroendocrine, autonomic and respiratory responses to aversive stimuli. Lesioning, stimulation and neurochemical studies have shown that the amygdala is a critical component in the expression, conditioning and extinction of fear and acute anxiety (Davis, 1992; LeDoux, 1995). The primary output from the amygdala originates from the central nucleus and terminates in the PAG, brainstem and hypothalamus (Killcross *et al*, 1997). The lateral and basolateral nuclei receive cortical and subcortical (predominantly from the thalamus) sensory inputs (McCool and Chappell, 2007; LeDoux, 1995). Killcross *et al.* (1997) determined that lesions of both the basolateral and central nuclei of the amygdala prevented the formation of an association between a conditioned stimulus and a punishment in a modified Geller-Seifter test. However, lesioning of the basolateral or central nucleus of the amygdala alone did not abolish the formation of an association as anticipated but altered the behavioural expression of the association demonstrating that these nuclei mediate specific components of fear-conditioning (Killcross *et al*, 1997).

The lateral amygdaloid nucleus of the rat receives extensive serotonergic innervation, however, the central and medial nuclei receive relatively little serotonergic innervation (Hensler, 2006). The effects of pharmacological manipulations of the serotonergic transmission in the amygdala have been investigated. The administration of the 5-HT_{1A} agonist 8-OH DPAT into the basolateral amygdala induced anxiogenic-like effects in both the Geller-Seifter (Hodges *et al*, 1987) and social interaction tests (Gonzalez *et al*, 1996)

suggesting that activation of the 5-HT_{1A} receptor in the amygdala may exacerbate fear responses. However, the nature of the threatening stimulus may also be important as the administration of 8-OH DPAT into the basolateral amygdala failed to influence anxiety-like behaviours in the elevated plus-maze (Gonzalez *et al*, 1996). Similarly, the administration of 5-HT_2 antagonists methysergide and ketanserine into the basolateral amygdala generated conflicting results in the Geller-Seifter task (anxiolytic; Hodges *et al*, 1987) and the elevated plus maze (anxiogenic; Zangrossi Jr. and Graeff, 1994) respectively. The intra-amygdala administration of the 5-HT_3 antagonist ondansetron has been reported to be anxiolytic in the social interaction test but not the Vogel conflict task (Higgins *et al*, 1991). Collectively, the data suggest that the role of serotonergic transmission in the amygdala in the mediation of anxiety may be receptor specific and may also be dependant upon the nature of the aversive stimulus.

Using the Spielberger's state trait anxiety inventory and a visual analogue scale, the effect of lesions to the left hemisphere amygdala in two human patients were investigated (Masaoka *et al*, 2003). In the basal resting state, both patients were reported to have high levels of state and trait anxiety (Masaoka *et al*, 2003). However, indices of state and trait anxiety were found to be reduced in the basal state following surgery (Masaoka *et al*, 2003). In anticipation of a shock administered following a warning, lesioning of the left hemisphere amygdala was found to reduce state anxiety (Masaoka *et al*, 2003). In addition, a reduction in respiratory rate and skin conductance response of the patients was reported when compared before and after surgery (Masaoka *et al*, 2003).

1.2.3. Septo-hippocampal system

In 1982, Gray and McNaughton proposed that anxiolytic drugs act upon a 'behavioural inhibition system' which involves the septo-hippocampal system (McNaughton and Gray, 2000). The hypothesis arose from the observation that lesions of the septo-hippocampus induce a behavioural phenotype similar to that of anxiolytic drugs (McNaughton and Gray, 2000). In this system, the septo-hippocampus serves as a comparator of inputs signalling the current state versus the predicted state of an event. In the event of a mismatch, which can be generated by aversive stimuli such as punished responding, reward omission and novel stimuli, output from the septo-hippocampal system facilitates behavioural inhibition in an attempt reconcile the mismatch.

A range of anxiolytic drugs (including buspirone, ethanol and imipramine) have been reported to impair the control of theta activity in the hippocampus, which originates from the pacemaker input of the medial septal area (McNaughton and Gray, 2000). As the occurrence of theta activity in the hippocampus is associated with arousal, this offers a possible link by which anxiolytic compounds may alter the expression of the anxious state.

The serotonergic innervations to the hippocampus are mediated by both the dorsal raphe nucleus (DRN) and the median raphe nucleus (MRN). The dorsal hippocampus is innervated exclusively by serotonergic neurons originating from the MRN. However, the ventral hippocampus receives afferent innervation from both nuclei (Azmitia and Segal, 1978). The potentiation of GABAergic transmission in the dorsal hippocampus elicits an anxiolytic effect in the majority of studies. In agreement, the administration of midazolam into the dorsal hippocampus is reported to be anxiolytic in the open field (Stefanski *et al*, 1993), the Vogel conflict task (Stefanski *et al*, 1993; Plaznick *et al*, 1994) and social interaction test (Gonzalez *et al*, 1998) but not the elevated plus-maze

(Gonzalez *et al*, 1998). However, the role of serotonergic transmission in the dorsal hippocampus appears to be dependant upon the nature of the stressor and the dose of the substance administered. The administration of 8-OH DPAT is reported to induce anxiogenic effects in the social interaction test (Andrews *et al*, 1994; File *et al*, 1996) and in the elevated plus-maze in maze experienced rats (File *et al*, 1996). However, studies have reported anxiolytic effects of 8-OH DPAT administered into the dorsal hippocampus in the open field (Stefanski *et al*, 1993), Vogel conflict task (Stefanski *et al*, 1993; Przegalinski *et al*, 1994), the ultrasonic vocalisation test (Schreiber and De Vry, 1993) and a passive avoidance task (Carli *et al*, 1993). The anxiolytic effects reported are typically in response to the administration of higher doses than those reported to elicit anxiogenic effects. The dose dependent effects may reflect the distinct contributions both pre and post-synaptic 5-HT_{1A} receptors to the behavioural effect at a particular dose or the diffusion of 8-OH DPAT to distal sites at higher doses (File *et al*, 2000).

The intra-ventral hippocampal administration of 8-OH DPAT has no effect in either the social interaction test or elevated plus-maze (Hogg *et al*, 1994; File and Gonzalez, 1996). However, the administration of the antagonist tertatolol into the ventral hippocampus is reported to elicit anxiolytic-like effects in the elevated plus-maze in animals naïve and experienced with the maze (File and Gonzalez, 1996). Using microdialysis, Rex *et al*. (2005) reported that exposure to the elevated plus-maze increased serotonin overflow in the ventral hippocampus. Therefore, the anxiolytic-like effect of tertatolol administration may reflect the endogenous serotonergic tone induced by the elevated plus-maze.

1.2.4. Periaqueductal grey

The periaqueductal grey (PAG) has been implicated as component in the circuitry which mediates the expression of fear behaviours in response to a range of stressors (Canteras *et al*, 2012). Delgado *et al*. (1954) showed that animals learn to respond in order to cease the stimulation of the dorsal PAG. Therefore, the stimulation of the dorsal PAG appears to an aversive stimulus in itself and has been investigated as a test of anxiety (Graeff, 2004). The systemic administration of the 5-HT synthesis inhibitor PCPA or the 5-HT receptor antagonist cyproheptadine facilitate the 'switching off' of the stimulation of the dorsal PAG suggesting an anxiogenic-like effect (Kiser *et al*, 1978b; Schenberg and Graeff, 1978). Conversely, the facilitation of serotonergic transmission induced by the systemic administration of 5-hydroxytryptophan (5-HTP), chlorimipramine, and the stimulation of the DRN all reduced 'switching-off' of the stimulation of the dorsal PAG suggesting an anxiolytic-like effect (Kiser *et al*, 1978a; Kiser *et al*, 1980). However, the systemic administration of chlordiazepoxide also reduced the 'switching-off' response (Schenberg and Graeff, 1978). In classical conflict tasks, the administration of benzodiazepines and 5-HT receptor antagonists are typically associated with anxiolytic-like effects and not opposing effects as was reported in response to the stimulation of the dorsal PAG.

This apparent contradiction was resolved by Graeff and Rawlins (1980). Rats were trained to press a lever for a food reward and subsequently divided into two groups. Each group was presented with a punished stimulus paired with the lever pressing response, however, one group received a mild foot-shock and the other received a brief stimulation of the dorsal PAG. Following a lesion of the lateral septum in both groups, punished responding in animals which received the mild-shock was increased. However, rats which received the mild

stimulation of dorsal PAG showed no increase in the rate of punished responding. Therefore, the results suggested that the dorsal PAG is a component of the brain defence system in which serotonin originating from the DRN acts to inhibit aversive responding. This circuit may be distinct from the behavioural inhibition system, in which serotonin facilitates inhibition (Graeff, 2004).

In addition to maintaining 'switching-off' responding, the stimulation of the dorsal PAG has been associated with an increase in flight, fight and aggressive postures in rats (Graeff, 2004). Similarly, Schmitt *et al*. (1986) reported that the administration of $GABA_A$ receptor antagonists into the dorsal PAG elicited aimless vertical jumps, alternating with periods of immobility and freezing. In humans, the stimulation of the dorsal PAG elicits feelings of fear, impending death and the desire to flee (reviewed by Graeff, 2004). Furthermore, patients showed palpitations, blushing of the face and neck and respiratory arrest or hyperventilation. Therefore, the dorsal PAG has been implicated in pathology of panic attacks due to the similarities in the symptoms presented in patients diagnosed with panic disorder.

1.2.5. Prefrontal cortex

An important aspect of anxiety is the subjective experience of the aversive event. The subjective experience is dependant on the ability of the individual to alter the onset, duration, intensity or termination of the aversive event (Maier and Watkins, 2010). The ventromedial prefrontal cortex (vmPFC) has been implicated in mediating the psychological effects of behavioural control. Animals received escapable or yoked inescapable tail shocks, with only one animal capable of terminating the shock via a wheel in the testing box (Amat *et*

al, 2005). Inescapable shocks induced a behavioural phenotype thought to reflect a persistent state of anxiety (Maier and Watkins, 1998). However, individuals subjected to escapable shocks did not exhibit this behavioural phenotype despite both animals receiving identical number of shocks of equal intensity and duration. Although inescapable stress activates the serotonergic neurons originating in the DRN more than equal controllable stress (Bland *et al*, 2003) the DRN is unlikely to process behavioural control. Anatomically, the lack of direct sensory and motor inputs in addition to the size of the DRN (11,500-15,191 serotonergic neurons in the rat; Vertes and Linley, 2008) suggest this nucleus does not have the required information or processing capacity to determine behavioural control. However, the DRN receives cortical inputs via glutamatergic innervations from the infralimbic and prelimbic areas of the vmPFC which terminate on GABAergic interneurons in the DRN (Vertes, 2004; Jankowski and Sesack, 2004; Celada *et al*, 2001). Therefore, Amat *et al*. (2005) investigated the role of the vmPFC in mediating behavioural control by locally administering the $GABA_A$ receptor agonist muscimol immediately prior to escapable and yoked inescapable stress. The administration of muscimol into the vmPFC prior to escapable stress evoked a prolonged increase in serotonin overflow in the DRN comparable to that observed in the animals exposed to the inescapable shocks (Amat *et al*, 2005). In addition, the intra-vmPFC administration of muscimol prior to escapable shocks increased the latency of shuttlebox escape (Amat *et al*, 2005) and reduced social investigation of a juvenile conspecific (Christianson *et al*, 2009) characteristic of yoked animals which received inescapable stress. Conversely, the administration of the $GABA_A$ receptor antagonist picrotoxin into the vmPFC ameliorated the behavioural effect of inescapable stress and induced a behavioural phenotype

comparable to animals which received escapable stress (Amat *et al*, 2008; Christianson *et al*, 2009).

1.2.6. Locus coeruleus

The role of noradrenaline in anxiety has been implicated by the finding that the α_2 adrenergic agonist clonidine has therapeutic value in the treatment of anxiety disorders (Hoehn-Saric *et al*, 1981; Uhde *et al*, 1989; Charney and Heninger, 1986). Conversely, the administration of yohimbine and piperoxam are reported to increase the subjective experience of fear and panic in both animals and humans (Iversen, 1984). In the startle response task, the administration of adrenergic agonists and antagonists are reported to be anxiogenic and anxiolytic respectively (Davis *et al*, 1979). Furthermore, the electrical stimulation of the locus coeruleus in animals is reported to induce anxiogenic behaviours in animals which are similar to anxiety in humans (Tanaka *et al*, 2000). This anxiogenic-like effect is blocked by lesions to the locus coeruleus and by noradrenergic antagonists (Uhde *et al*, 1984). In response to a variety of stressors, Tanaka *et al*. (2000) reported that noradrenergic overflow is increased in the hypothalamus, the amygdala, locus coeruleus, cerebral cortex and hippocampus. These increases were dependent on the controllability and predictability of the stressor. Therefore, the locus coeruleus has been implicated in the mediation of the fundamental 'alarm reaction' to aversive stimuli (Redmond, 1986; Charney and Heninger, 1986).

1.3. Brain serotonergic pathways as a target of anxiolytic drugs

1.3.1. The serotonergic system

Historically, the name serotonin described a “serum” factor that affected blood vessel “tonus” causing vasoconstriction and increasing gut motility (reviewed by Jacobs and Azmitia, 1992). In the mid-twentieth century, the endogenous compound was isolated, synthesised and the molecular formula elucidated. Serotonin was proposed as a neurotransmitter shortly after as it was found in varying concentrations throughout the mammalian CNS. Using the Falck-Hillarp histochemical fluorescence technique, Dahlstrom and Fuxe (1964) identified the localisation of serotonergic cell bodies and axon terminals in the rat brain. The serotonergic system of the CNS comprises eight distinct nuclei located at the midbrain (Lechin *et al*, 2006). The serotonergic nuclei were labelled B1 to B9 in order of their position along the saggital axis (Dahlstrom and Fuxe, 1964). With the exception of the B9 neurons (referred to as the supralemniscal nucleus), the serotonergic nuclei are located along at the midline of the brain. Therefore, the nuclei were referred to as the “raphe” nuclei which means “seam” (Vertes and Linley, 2008). The alpha-numeric nomenclature of the nuclei was replaced by names which reflect the anatomical location, however, the exception are B9 neurons which have retained the original nomenclature (reviewed by Vertes and Linley, 2008).

The raphe pallidus (B1), raphe obscurus (B2), raphe magnus (B3) and parts of the lateral reticular formation (B1/B3) are referred to as the caudal raphe complex and are located in the lower pons and the medulla. Projections from these nuclei typically descend to the brainstem and spinal cord and are implicated in the circuitry of cardiovascular, respiratory and ingestive control. The rostral raphe complex, which is predominantly located in the pons, is comprised of the caudal linear nucleus (B8), the DRN (B6 and B7) and the median raphe nucleus (MRN; B8 and B5) and are the origin of ascending

serotonergic projections to the forebrain (Hornung, 2003). The major site of serotonin synthesis in the central nervous system (CNS) is the DRN and, to a lesser extent, the MRN in the midbrain (Pineyro and Blier, 1999). There are three major ascending serotonergic pathways to the forebrain consisting of highly collateralised projections which extensively innervate the forebrain. In addition to anxiety, serotonergic transmission has been implicated in mediating the sleep-wake cycle, food intake and sexual behaviour (Barnes and Sharp, 1999).

1.3.2. Role of serotonergic transmission in anxiety

The neurotransmitter serotonin has been implicated in the mediation of anxiety for a long time. Historically, the benzodiazepines were reported to reduce the turnover and release of catecholamines and indolamines such as serotonin in the brain (Wise *et al*, 1972). Therefore, it was proposed that the anxiolytic effect of the benzodiazepines was, in part, due to the suppression of serotonergic transmission. In addition, several lines of clinical evidence support the hypothesis that serotonin is an important mediator of anxiety. The administration of the 5-HT agonist m-chlorophenylpiperazine (mCPP) is anxiogenic in patients with panic disorder (Charney *et al*, 1987), obsessive compulsive disorder (Zohar *et al*, 1987) and at high doses in healthy individuals (Charney *et al*, 1987). Similarly, the serotonin re-uptake inhibitior fenfluramine is reported to be anxiogenic in patients diagnosed with panic disorder, social phobia and healthy individuals (Targum, 1990; Tancer *et al*, 1994). In agreement, serotonin metabolites are reduced in the cerebrospinal fluid of patients with impulsivity disorders which show reduced aversive processing and behavioural disinhibition (Linnoila and Virkkunen, 1992). However, the administration of L-tryptophan and 5-HTP, the metabolic precursors of

serotonin, have been reported to induce sedation and anxiolysis (Charney *et al*, 1987; Nutt and Cowen, 1987; Westenberg and den Boer, 1989). In addition, the clinical efficacy of the selective reuptake inhibitors in the treatment of the anxiety disorders would support the hypothesis that serotonin mediates anxiety (Lydiard *et al*, 1996). However, the clinical evidence appears contradictory in the specific role that serotonin plays in the mediation of anxiety.

Early work on the reduction in serotonergic transmission found that the serotonin antagonists cinanserin, cyproheptadine, methysergide and lysergic diethylamide released response suppression in rats. Similarly, the depletion of serotonin using p-chloroamphetamine produced a marked increase in punished responding (reviewed by Iversen, 1984). Lesioning of the serotonergic system have been reported following the administration of the toxin 5,7-dihydroxytryptamine (5,7-DHT). When administered into the ventral tegmental area, 5,7-DHT is reported to release punished responding in a Geller-Seifter task and impair the acquisition of response suppression to the shock (Tye *et al*, 1977). The 5,7-DHT lesion of the ventral tegmental area is reported to deplete serotonin in the hypothalamus, limbic and cortical structures but leaves the dorsal serotonergic projections to the striatum relatively intact. The lesion did not induce a stimulation of motor activity and therefore suggests that the effect on response suppression was due to an anxiolytic-like effect (Tye *et al*, 1979). Therefore, the suppression of the serotonergic system appears to mimic the anxiolytic-like effects of the benzodiazepines in punished conflict tasks. Historically, serotonin has been implicated in the mediation of aversive processing (Deakin and Graeff, 1991; Kranz *et al*, 2010) and behavioural inhibition (Soubrié, 1986).

The pathology of depression is associated with the reduction in serotonergic transmission and is characterised by reduced behavioural vigor and enhanced aversive processing (Deakin and Graeff, 1991; Eshel and Roiser, 2010; Clark *et al*, 2009). Given that anxiety and depression are often expressed as co-morbidities in patients and the clinical efficacy of the SSRIs in the treatment of both depression and many anxiety disorders, it is difficult to reconcile that serotonin is simply anxiogenic. This apparent contradiction was investigated by Graeff *et al*. (1996) who proposed that serotonin plays a dual role in the mediation of anxiety.

As discussed previously, serotonin suppresses the responses which terminate the electrical stimulation of the PAG in a similar manner to the benzodiazepines. Therefore, Graeff *et al*. (1996) proposed that distinct pathways and receptor subtypes mediate the anxiolytic and anxiogenic effects of serotonin. Serotonergic projections originating in the DRN terminate in the amygdala and prefrontal cortex and facilitate active escape or avoidance behaviours in response to potential or distal threats (*figure 1.2.*). These strategies require learning and are thought to relate to conditioned fear, anticipatory anxiety and generalised anxiety disorder. In contrast, the serotonergic innervations from the DRN to the PVN and PAG matter inhibit the primitive fight/flight responses triggered in response to proximal threat, acute pain or asphyxia that may relate to panic disorders (*figure 1.2.*). In the absence of behavioural control, an acute stressor can become a chronic stressor. In response to chronic stress, as opposed to acute stress, Graeff *et al*. (1996) suggest the projections from the MRN to the hippocampus promote resistance to the stressor by disconnecting the stressor from social and appetitive neurobiological processes. Therefore,

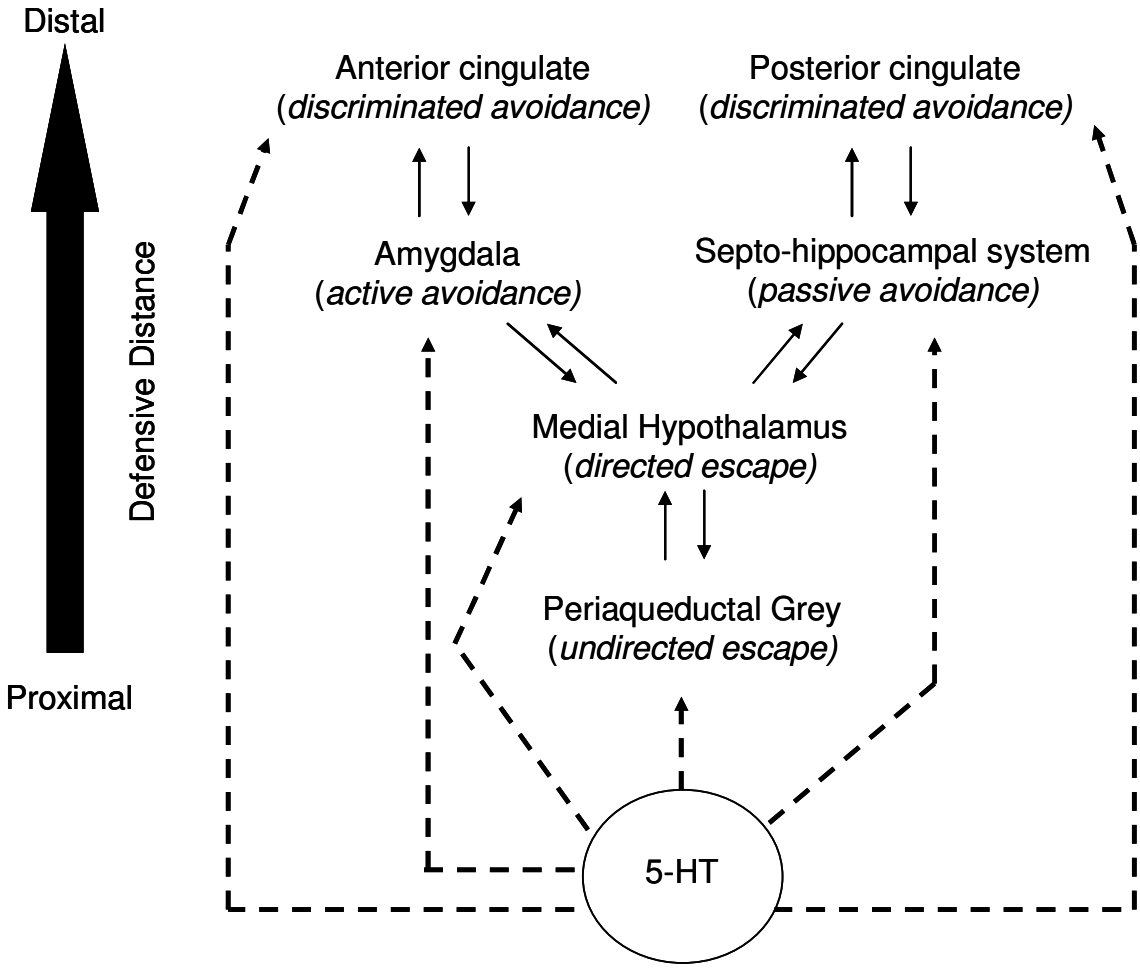

Figure 1.2. –The serotonergic system plays a modulatory role at each level of the proposed neuronal hierarchy of defensive behaviours. Serotonergic afferent projections terminate in higher order centres such as the amygdala, the septo-hippocampal system, the cingulate and prefrontal cortices to facilitate avoidance behaviours in response to distal or more cognitively complex threats. However, the serotonergic afferents which terminate in the periaqueductal grey and hypothalamus inhibit the primitive fight/flight responses evoked by proximal threats, asphyxia and acute pain. Adapted from Graeff *et al*, 1996 and McNaughton and Corr (2004).

this process would allow for the individual to continue functioning normally despite on-going aversive events, however, its dysfunction would likely contribute to the pathology of depression.

More recently, the role of serotonin in both valence (reward versus punishment) and activity (invigoration or inhibition) has been expanded upon (Boureau and

Dayan, 2011). Serotonin is most commonly associated with the inhibition of punished responses therefore coupling negative affect with behavioural inhibition (Soubrié, 1986). However, Boureau and Dayan (2011) have proposed that the two effects are dissociable as serotonin has been implicated in both behavioural inhibition (Graeff *et al*, 1996; Gray and McNaughton, 2003) and active avoidance (Bland *et al*, 2003) in response to adverse events. Boureau and Dayan (2011) propose a model where serotonin acts cooperatively with dopamine to maximise rewards and minimise the likelihood of aversive events in the most energy efficient manner. In this model, Boureau and Dayan (2011) suggest that dopamine and serotonin represent opposite ends in the spectrum of affective valence and motor activity. Therefore serotonin, in conjucture with dopamine, may be able to mediate both active responses (such as escape or avoidance) and behavioural inhibition (such as freezing) in response to aversive stimuli. In addition, Cools *et al*. (2011) have suggested that the tonic release of serotonin signals the anticipated valence associated with future events (i.e. the likelihood of aversive outcomes) whilst the phasic release of serotonin acts to signal the valence of experienced events (i.e. the outcome of the event). Therefore, serotonin may act as a predictive index of aversive events and this may play an important role in the formation of Pavlovian associations in response to aversive events.

1.4. Dorsal raphe nucleus

In rats, the DRN (B7 and B6) is situated in the pons directly ventral to the cerebral aqueduct which traverses the PAG (*figure 1.3.*). Along the coronal axis, the DRN is subdivided into lateral (or lateral wings) and medial subdomains (*figure 1.3.*). The latter is further compartmentalised into the dorsomedial, ventromedial and interfascicular domains (*figure 1.3.*). The medial subdivisions

extend through the rostral and middle aspects along the sagittal plane, however, are absent in the caudal domain (Michelsen *et al*., 2007). The majority of neurons which comprise the DRN synthesise the neurotransmitter serotonin. In cats, the estimated 24,000 serotonergic neurons reflect 70% of the total neuronal population of the DRN (reviewed by Jacobs and Azmitia, 1992). Similar estimates have been reported in the rat DRN and human DRN (Descarries *et al*, 1982; Yasufuku-Takano *et al*, 2008; Baker *et al*, 1991). In the rostral and medial domains, the serotonergic neurons of the DRN are densely populated at the midline and become increasingly diffuse in the lateral wings (Vertes and Linley, 2008).

The electrophysiological properties of the serotonergic neurons of the DRN were originally characterised in anaesthetised rats and cats (Aghajanian *et al*, 1978; Jacobs and Azmitia, 1992). The serotonergic neurons were hyperpolarised by the focal application of serotonin, which is mediated by the inhibitory 5-HT_{1A} autoreceptor (Sprouse and Aghajanian, 1986; Sotelo *et al*, 1990). In addition, a proportion of non-serotonergic neurons also express the 5-HT_{1A} autoreceptor (Kirby *et al*, 2003; Yasufuku-Takano *et al*, 2008). In the DRN, the 5-HT_{1A} autoreceptor is a G-protein coupled receptor linked to potassium channels which induce the hyperpolarisation of the membrane by increasing potassium conductance in response to increased extracellular serotonin (Aghajanian and Lakosi, 1984; Adell *et al*, 2002). A secondary inhibitory mechanism is also mediated by the activation of 5-HT_{2A} receptors which are expressed on the GABAergic inhibitory inputs to the serotonergic neurons (Lechin *et al*, 2006; Martin-Ruiz *et al*, 2001a).

Until the mid-seventies, the DRN was considered exclusively serotonergic, however, additional neurotransmitters and peptides have been reported. Dopaminergic neurons are located in ventromedial and dorsorostral regions of

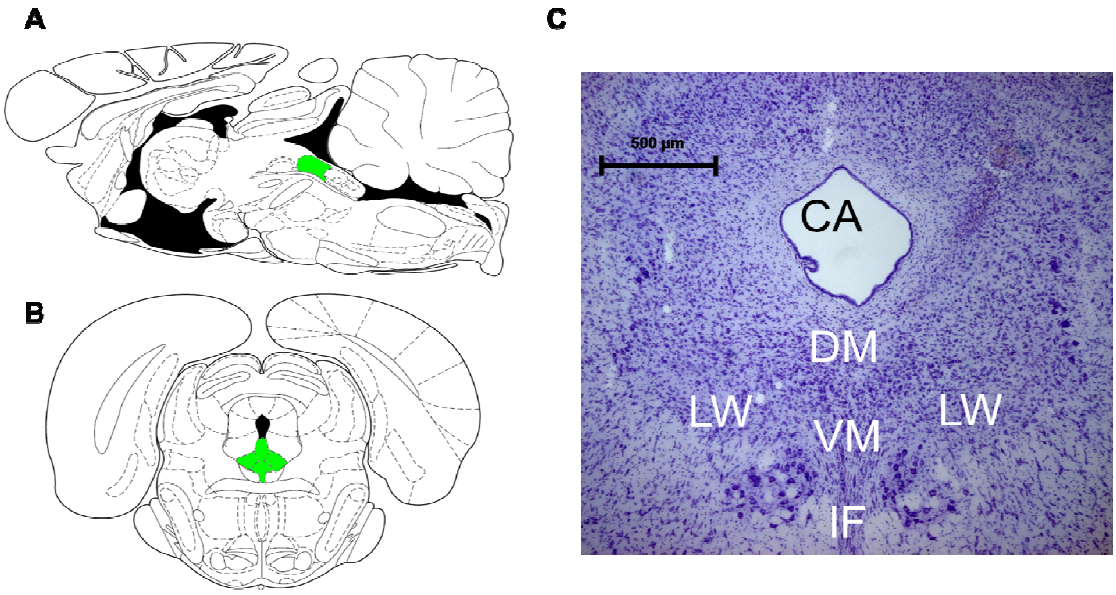

Figure 1.3. –The localisation of the DRN in the sagittal and coronal plane of the rat brain and the subdivisions of the DRN. In the sagittal section (lateral = -0.1 mm), the DRN (shown in green) is situated in the pons and is bordered rostrally by the trochlear nucleus and caudally by the dorsal tegmental nucleus (**panel A**). The DRN is situated at the midline and is located ventral to the cerebral aqueduct (CA) and dorsal to the medial longitudinal fasciculus in the coronal plane (AP = -7.8 mm; **panel B**). The DRN, in the coronal plane, is subdivided into the medial and lateral domains (LW). The former is further subdivided into the dorsomedial (DM), ventromedial (VM) and interfascicular domains (IF; AP = -7.8 mm; **panel C**). Figures adapted from Paxinos and Watson (1998).

the DRN (Ochi and Schimizu, 1978; Stratford and Wirtshafter, 1990). The expression of both the phosphate activated glutaminase and vesicular glutamate transporter-3 (VGLUT3) in the serotonergic and non-serotonergic neurons of the DRN suggest that the excitatory amino acid glutamate is synthesised in the DRN (Kaneko *et al*, 1990; Mintz and Scott, 2006; Jackson *et al*, 2009). Similarly, the expression of nitric oxide synthase in the serotonergic

neurons of the DRN may also suggest that that nitric oxide may act as a neurotransmitter in the DRN (Dun *et al*, 1994).

A number of neuroactive peptides have been reported to have influence the serotonergic transmission of the DRN. Immunoreactivity for corticotrophin releasing factor (CRF) has been found in both the dorsomedial and lateral wings of the DRN and co-localises with the serotonergic neurons (Commons *et al*, 2003). Anterograde tracing has identified that CRF-containing neurons of the DRN predominantly project to the amygdala and the dorsal hypothalamic area (Commons *et al*, 2003). In addition, there is evidence to suggest the expression of neuropeptide Y, substance P and galanin in the DRN (Pau *et al*, 1998; Baker *et al*, 1991; Le Maître *et al*, 2013).

1.4.1. The serotonergic neurons of the dorsal raphe nucleus

Typically, the serotonergic neurons of the DRN are densely packed, large (30-40 μm) and fusiform in shape (Vertes and Linley, 2008). In contrast, the serotonergic neurons of the MRN are more diffusely localised and are relatively small (10-12 μm) and oval (Vertes and Linley, 2008). Baker *et al*. (1990) reported morphologically distinct populations of serotonergic neurons in the human DRN. In agreement, Steinbusch *et al*. (1981) described the morphology of the serotonergic neurons in the rat DRN as small round, medium-sized fusiform, bipolar or large fusiform. Therefore, the serotonergic neurons of the DRN are heterogeneous and their topographical arrangement may reflect distinct functional roles (Abrams *et al*, 2004). Similarly, electrophysiological studies in the rat have described two distinct populations, termed type I (typical) and type II (atypical), based on firing properties and localisation (Abrams *et al*, 2004).

In addition, the mechanisms by which serotonin is released from the serotonergic neurons appears to be complex. Axonal varicosities containing vesicles suggest that the neurons store and release serotonin at discrete synapses (Adell *et al*, 2002). Serotonin release from the raphe nuclei is predominantly induced by action potential propagation, which is both tetrotoxin sensitive and Ca^{2+}-dependent (Adell *et al*, 2002). However, a volume or paracrine release mechanism may also exist which may be mediated by depolarisation-induced reversal of the serotonin transporter (Richerson and Wu, 2003). The type D axons of serotonergic neurons originating in the DRN are typically small with fine varicosities (Michelsen *et al*, 2007). The presence of vesicles at varicosities lacking synaptic junctions implies serotonergic type D axons predominantly utilise a paracrine release mechanism as opposed to a synaptic mechanism (Bunin and Wightman, 1999; Adell *et al*, 2002).

1.4.2. The afferent innervations of the dorsal raphe nucleus

The afferent innervations of the DRN encompass a number of neurotransmitters and peptides originating from nuclei implicated in the expression of the anxious state. Initially, studies using horseradish peroxidase and wheat germ agglutinin-horseradish peroxidase demonstrated an extensive afferent innervation from the lateral hebenula, in addition to minor descending afferents from the bed nucleus of stria terminalis, the horizontal band of Broca, the prefrontal cortex, the preoptic area and posterior hypothalamus (Aghajanian and Wang, 1977; Kalén *et al*, 1985).

The previously described afferents were refined and added to by Peyron *et al*. (1998) using anterograde and retrograde tracers. The administration of cholera toxin b into the DRN demonstrated cortical afferents originating from the orbital,

cingulated, infralimbic, dorsal peduncular and insular cortices. Using anterograde tracing, the projections from the prelimbic cortex were found to terminate in the dorsal region of the middle DRN and lateral wings. The DRN receives afferent innervations from the lateral, dorsal and posterior hypothalamic areas. In particular, there is a large afferent innervation from the arcuate nucleus, the ventromedial and dorsomedial hypothalamic nuclei and a moderate innervation from the parvocellular hypothalamic nucleus. In addition, there was a small innervation from the tuberomammillary nucleus to the DRN which is thought to be histaminergic (*figure 1.4.*). Additional afferent innervations were found to originate in the central nucleus of the amygdala, the ventral pallidum, the claustrum, the lateral and medial septum, the lateral, ventral and medial parts of the bed nucleus of stria terminalis, the medial and lateral preoptic areas, the medial preoptic nucleus and the medial portion of the lateral habenula (*figure 1.4.*).

The pharmacology of many of these afferent innervations has been characterised using electrophysiological and neurochemical techniques. Interconnectivity of the raphe nuclei has been reported in both the adult brain of the cat and rat and is serotonergic in nature (Jacobs and Azmitia, 1992). The principle serotonergic afferent innervation to the DRN originates from the MRN, which in turn is reciprocally innervated by the DRN (Jacobs and Azmitia, 1992; *figure 1.4.*). The reciprocal innervations from the serotonergic neurons mediate the activation of 5-$HT_{2A/2C}$ receptors which, in turn, facilitate the GABAergic inhibition of the serotonergic neurons (Liu *et al*, 2000; Serrats *et al*, 2005). Afferent innervation originating from the medial preoptic area, the lateral hypothalamic nuclei, the VTA and the substantia nigra is dopaminergic in nature (Adell *et al*, 2002; *figure 1.4.*). In agreement, the systemic, but not local,

administration of D1 and D2 receptor agonists facilitates serotonergic activity in the DRN (Martín-Ruiz *et al*, 2001b). However, the afferent innervations from the arcuate and dorsomedial hypothalamic nuclei are not dopaminergic but are thought mediate the release neuropeptides and histamine (Jacobs and Azmitia, 1992; *figure 1.4.*).

Excitatory noradrenergic afferent innervation of the DRN originates from the locus coeruleus and varies with arousal states (Lechin *et al*, 2006; *figure 1.4.*). This excitation is mediated by the activation of the α_{1B} adrenoceptor (Day *et al*, 1997). However, the expression of the α_2-adrenoceptor suggests that noradrenaline may also inhibit the serotonergic neurons of the DRN (Strazielle *et al*, 1999). The glutamatergic innervation of the DRN originates from the prefrontal cortex, hypothalamic nuclei, lateral habenula and the PAG. The release of glutamate activates AMPA and NMDA receptors in the DRN and therefore these afferents are excitatory in nature (Liu *et al*, 2002; Gartside *et al*, 2007). However, these glutamatergic afferents, in particular those originating from the vmPFC and lateral habenula, can mediate the inhibition of the serotonergic neurons indirectly via excitation of the GABAergic interneurons of the DRN (Celada *et al*, 2002; Gartside *et al*, 2007; *figure 1.4.*).

Although both raphé nuclei contain a small number of GABAergic interneurons, the majority of inhibitory afferents originate at distal sites (Adell *et al.*, 2002). GABAergic afferents originate from the hypothalamus, the substantia nigra, the VTA and ventral PAG (Gervasoni *et al*, 2000; *figure 1.4.*). In addition, a combined study using retrograde tracing and glycine immunohistochemistry identified that the main glycinergic innervation to the DRN in rats originates from the ventral and ventrolateral PAG (Rampon *et al*, 1999; *figure 1.4.*).

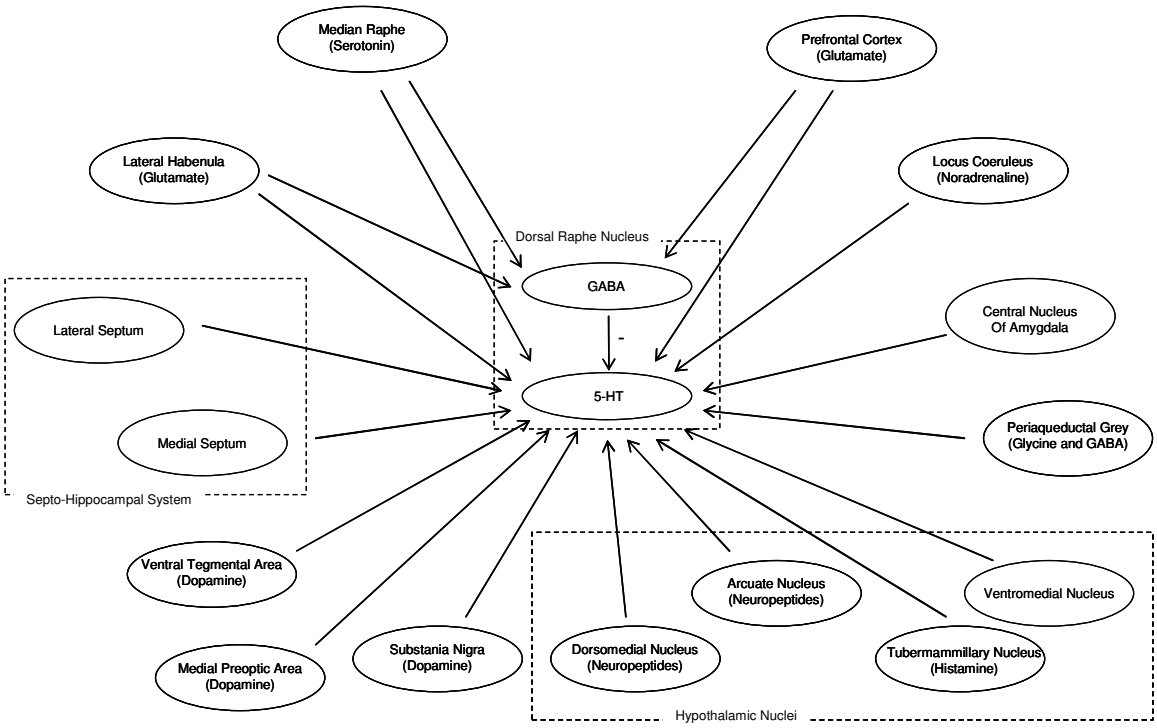

Figure 1.4. –Schematic representation of the afferent projections which terminate in the dorsal raphe nucleus. The afferent innervations which terminate in the DRN originate from a diverse array of limbic and cortical centres previously implicated in the neuronal processes which underpin the generation of anxiety. Such centres include the prefrontal cortex, locus coeruleus, the amygdala, the periaqueductal grey, the hypothalamic nuclei and the septo-hippocampal system. In addition, the afferent innervations originating from the median raphe nucleus, the lateral habenula and prefrontal cortex have been reported to indirectly inhibit the neuronal activity of the serotonergic neurons via the excitation of GABAergic interneurons. Adapted from Vertes and Linley (2008) and Lechin *et al*. (2006).

CRF containing neurons terminate on both the serotonergic neurons and GABAergic interneurons of the DRN (Kirby *et al*, 2008; Waselus *et al*, 2005). The CRF_1 receptor, which is the high affinity receptor for CRF, is expressed on both serotonergic and GABAergic neurons of the DRN. Conversely, the low affinity CRF_2 receptor is exclusively expressed by the serotonergic neurons of the DRN. The effect of CRF in the DRN is bi-phasic, with low concentrations mediating the inhibition of serotonergic neurons and higher concentrations facilitating serotonergic transmission from the DRN (Lukkes *et al*, 2008). At low

concentrations, CRF facilitates the inhibition of the serotonergic neurons by the activation of CRF_1 receptors expressed by the GABAergic interneurons (Kirby *et al*, 2008). However, at high concentrations CRF mediates the excitation of the serotonergic neurons via the activation of the CRF_2 receptors (Kirby *et al*, 2008).

The DRN and MRN contain opioids such as dynorphin and enkephalin (Adell *et al*, 2002). Indeed, the attenuation of serotonin release from the midbrain raphé in response to morphine administration, which binds μ-opioid receptors, may result from disinhibition of GABAergic afferents or inhibition of excitatory inputs (Adell *et al*, 2002). The stimulation of serotonin release from the DRN induced by nicotine administration suggests there may be nicotinic acetylcholine receptors in the midbrain nuclei (Mihailescu *et al*, 1998). Cholinergic afferents from the brain stem originate from superior vestibular nucleus and terminate in the DRN via the medial longitudinal fasciculus (Jacobs and Azmitia, 1992). In addition, previous studies have reported that serotonergic release from the DRN and MRN may be regulated by histamine, nitric oxide, substance P, CRF and galanin may also modulate serotonergic activity (Adell *et al*, 2002).

1.4.3. The efferent projections of the dorsal raphe nucleus

With few exceptions, the DRN and the MRN generally project to distinct non-overlapping sites in the forebrain. The MRN can be characterised as a midline/paramidline projection system (Vertes and Linley, 2008). The MRN innervates few laterally situated structures with the exception of modest innervations to the perirhinal and entorhinal cortices (Vertes, 1991). In contrast, the DRN projects to those regions and structures not innervated by the MRN. Despite the relatively small numbers of neurons contained within the DRN, the

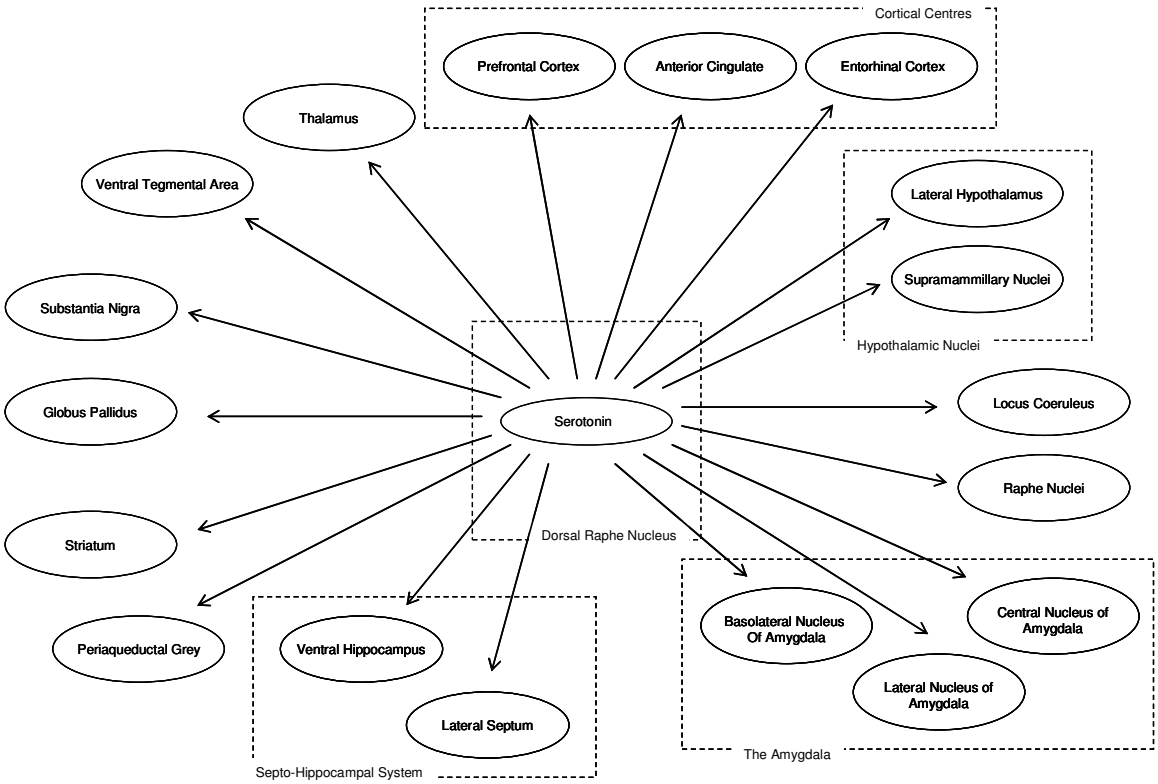

Figure 1.5. –Schematic representation of the efferent serotonergic projections which originate in the dorsal raphe nucleus. The efferent serotonergic projections which originate in the DRN terminate in both limbic and cortical centres implicated in the neuronal processes which underpin the generation of anxiety. Such centres include the prefrontal cortex, locus coeruleus, the amygdala, the periaqueductal grey, the hypothalamic nuclei and the septo-hippocampal system. Adapted from Vertes and Linley (2008).

highly collateralised nature of these ascending projections leads to widespread innervations of the forebrain (Jacobs and Azmitia, 1992; *figure 1.5.*).

The DRN innervates the forebrain via three projection pathways – the ventral, medial and dorsal ascending pathways (Michelsen *et al*, 2007). The dorsal ascending pathway originates in the medial and rostral DRN and innervates the striatum and globus pallidus (Michelsen *et al*, 2007; *figure 1.5.*). The substantia nigra is the main target of the medial ascending pathway (*figure 1.5.*). The projections of the medial ascending pathway originate in the rostral DRN (Michelsen *et al*, 2007). The most extensive innervations of the forebrain

originate from the ventral ascending pathway and terminate in numerous regions implicated in the mediation of anxiety (*figure 1.5.*). The pathway ascends ventrolaterally and then turns rostrally to enter the medial forebrain bundle (Michelsen *et al*, 2007). The ventral ascending pathway, which includes afferents from all three mesencephalic raphe nuclei, innervates thalamic and hypothalamic nuclei, the habenula, the septum, the amygdala, cortical centres and the hippocampus (Moore *et al*, 1978; Azmitia and Segal, 1978; *figure 1.5.*).

Using the anterograde tracer *Phaseolus vulgaris* leuccoagglutinin, the terminal domains of the ascending projections have been further refined (Vertes and Kocsis, 1994; Vertes, 1991). In the hypothalamus, projections from the DRN terminate in the lateral and supramammillary nuclei (Vertes, 1991; *figure 1.5.*). The efferent projections to the amygdala are extensive and innervate the central, lateral, basolateral, basomedial nuclei and the amygdalo-piriform transition zone (Vertes, 1991; *figure 1.5.*). In addition, innervation of the septo-hippocampal system was predominantly found in the medial lateral septum and was only moderate in the hippocampus (Vertes, 1991; *figure 1.5.*). Efferent projections to cortical nuclei include the anterior cingulate, prelimbic and infralimbic cortices of the prefrontal cortex and the entorhinal cortex (Vertes, 1991; *figure 1.5.*).

The DRN projects to the dopaminergic cell groups of the VTA and substantia nigra. In addition, the DRN innervates many of the terminal domains of the VTA and substantia nigra such as the dorsal and ventral striatum, the basal nuclei of the amygdala and medial prefrontal cortex (Vertes, 1991). Therefore the serotonergic projections from the DRN influence both the origins and terminal domains of the nigrostriatal and mesolimbic dopaminergic systems (Vertes and Linley, 2008). The dopaminergic neurons of the DRN terminate in the nucleus

accumbens, the lateral septum and to a lesser extent the caudate putamen (Stratford and Wirtshafter, 1990).

In addition, the serotonergic projections from the DRN have also been found to innervate regions of the midbrain, pons and medulla. These include the locus coeruleus, the central linear nucleus, the median raphe, the raphe magnus and raphe obscurus (Vertes and Kocsis, 1994; *figure 1.5.*). Due to the expansive distribution of the efferent projections originating from the DRN it is unsurprising that the DRN has been implicated in range of neuronal processes such as arousal, feeding, emotion, motivation, thermoregulation and cognitive processes (Jacobs and Azmitia, 1992)

1.4.4. Role of the dorsal raphe nucleus in anxiety

Early work showed that the iontophoretic application of 5-HT into the DRN inhibits neuronal excitation of the serotonergic neurons (Wang and Aghajanian, 1977). In agreement, Thiebot *et al*. (1984) reported that rats trained to press a lever for a food reward also received a foot shock with the presentation of the 'lights-off' conditioned stimulus. However, no shock was administered in the 'light-on' phase of the trial. 5,7-DHT lesions of the DRN induced a release of response suppression in the 'lights off' phase suggesting an anxiolytic-like effect. Similarly, the intra-DRN administration of GABA has been reported to release punished responding without reducing responding in the unpunished phase (Soubrie *et al*, 1981). In agreement, the systemic administration of clonazepam, chlordiazepoxide and midazolam depresses the firing of the serotonergic neurons of the DRN (Laurent *et al*, 1983).

Pharmacological manipulation of GABAergic inhibition in the DRN has been reported in a range of behavioural tests of anxiety in rodents. In rats naïve to

the elevated plus-maze, the administration of midazolam increased the time spent exploring the open runways of the plus-maze and the duration of time spent in social interaction in the social interaction test (Gonzalez and File, 1997). In the absence of any overt change in locomotor activity, these outcomes reflect an anxiolytic-like effect (Gonzalez and File, 1997). The intra-DRN administration of the benzodiazepine ligand flumazenil in rats previously exposed to the elevated plus-maze produced anxiolytic-like effects in both the social interaction test and elevated plus-maze (Gonzalez and File, 1997). The administration of the $GABA_A$ receptor agonist muscimol into the DRN increased social interaction and locomotor activity (Higgins *et al*, 1988). In agreement, muscimol was reported to increase punished responding in the Vogel-conflict task (Higgins *et al*, 1988). In mice, the intra-DRN administration of the benzodiazepine diazepam increased the latency of time to transition from the light to dark sections and the total time spent in the light compartment of the light-dark box (Costall *et al*, 1989). In the Geller-Seifer conflict task, the intra-DRN administration of chlordiazepoxide is reported to produce anxiolytic-like effects when tested to extinction (Thiebot *et al*, 1980; Thiebot *et al*, 1982). The evidence suggests that an increase, either via activation or positive allotsteric modulation, of the GABAergic inhibition in the DRN induces anxiolytic-like effects in behavioural tests of anxiety.

Using similar methods, the role of inhibitory 5-HT_{1A} autoreceptors expressed in the DRN has been investigated. In the social interaction test, intra-DRN administration of the 5-HT_{1A} agonists 8-OH DPAT, ipsapirone and buspirone were reported to be anxiolytic in the social interaction test (Higgins *et al*, 1988; Hogg *et al*, 1994). When investigated in the light dark box, the administration of buspirone into the DRN was reported to evoke anxiolytic-like effects in mice

(Costall *et al*, 1988) but not in rats (Carli *et al*, 1989). However, these reported species-dependent effects may be due to differences in the administered dose. When tested in the elevated plus-maze, the intra-DRN administration of 8-OH DPAT is reported to be anxiolytic in rats previously exposed to the elevated plus-maze but not those naïve to the apparatus (File and Gonzalez, 1996). The administration of 8-OH DPAT, ipsapirone, buspirone and gepirone into the DRN were all reported to induce anxiolytic-like effects in the Vogel-conflict task (Higgins *et al*, 1988; Higgins *et al*, 1992; Cervo *et al*, 2000). In agreement, the local administration of 8-OH DPAT into the DRN evoked anxiolytic-like effects in both the Geller-conflict and ultrasonic vocalisation tests (Schreiber and De Vry, 1993; Remy *et al*, 1996). Therefore, intra-DRN administrations of 5-HT_{1A} receptor agonists have been reported to increase anxiolytic-like behaviours in rodents.

Using immunohistochemistry, the expression of immediate early gene c-fos has been reported to be increased in the DRN in response to a variant of aversive stimuli (Cullinan *et al*, 1995). In addition, electrophysiological evidence in the cat rostral DRN has identified that the activity of the neurons is influenced by a range of psychological, metabolic and physical stressors (Chaouloff *et al*, 1999). Therefore the effect of stress on the serotonergic neurons of the DRN may be to promote arousal (Jacobs and Fornal, 1991; Jacobs and Azmitia, 1992). In agreement the presentation of an aversive stimulus facilitates serotonin overflow in the terminal domains of the projections originating from the DRN (Rueter and Jacobs, 1996; Adell *et al*, 1997; Bland *et al*, 2003). However, Rueter and Jacobs (1996) reported an increase in serotonin overflow in the prefrontal cortex in response to non-aversive stimuli such as the presentation of

food. Therefore, an increase in serotonin overflow may reflect an increase in arousal which is an important response in the mediation of anxiety.

1.4.5. Characterisation of the glycinergic inhibition of the serotonergic neurons of the dorsal raphe nucleus

Historically, the strychnine-sensitive glycine receptor has been associated with the mediation of inhibition in the spinal cord, however, the ubiquitous expression of the glycine receptor subunits in the forebrain, in particular the β subunit, suggests the receptors may play a more complex role in behaviour than had previously been assumed (Malosio *et al*, 1991). In agreement, recent studies have implicated a role for the strychnine-sensitive glycine receptor in the hippocampus (Keck *et al*, 2008; Eichler *et al*, 2009), nucleus accumbens (Ericson *et al*, 2006; Chau *et al*, 2010), amygdala (McCool *et al*, 2003; McCool and Chappel, 2007) and VTA (Li *et al*, 2012). A recent immunohistochemical study investigating the expression of the strychnine-sensitive glycine receptor in the human brain has identified abundant expression in the DRN (Waldvogel *et al*, 2010). In addition, the presence of strychnine-sensitive glycine receptors in the rodent DRN was originally suggested by an electrophysiological investigation. Thus, the iontophoretic application of glycine to the DRN evoked a suppression of firing of the serotonergic neurons in the DRN which was reversed by strychnine in anaesthetised rats (Gallager and Aghajanian, 1976). In agreement, Malosio *et al.* (1991) identified the presence of RNA encoding the α2 and beta subunits in the DRN of the adult rat brain. More recently, inhibitory neurotransmission has been characterised in slices of the mouse DRN (Maguire *et al*, 2013; *figure 1.6.*). In voltage-clamped serotonergic neurons, the majority of minature inhibitory post-synaptic currents were abolished by the antagonist

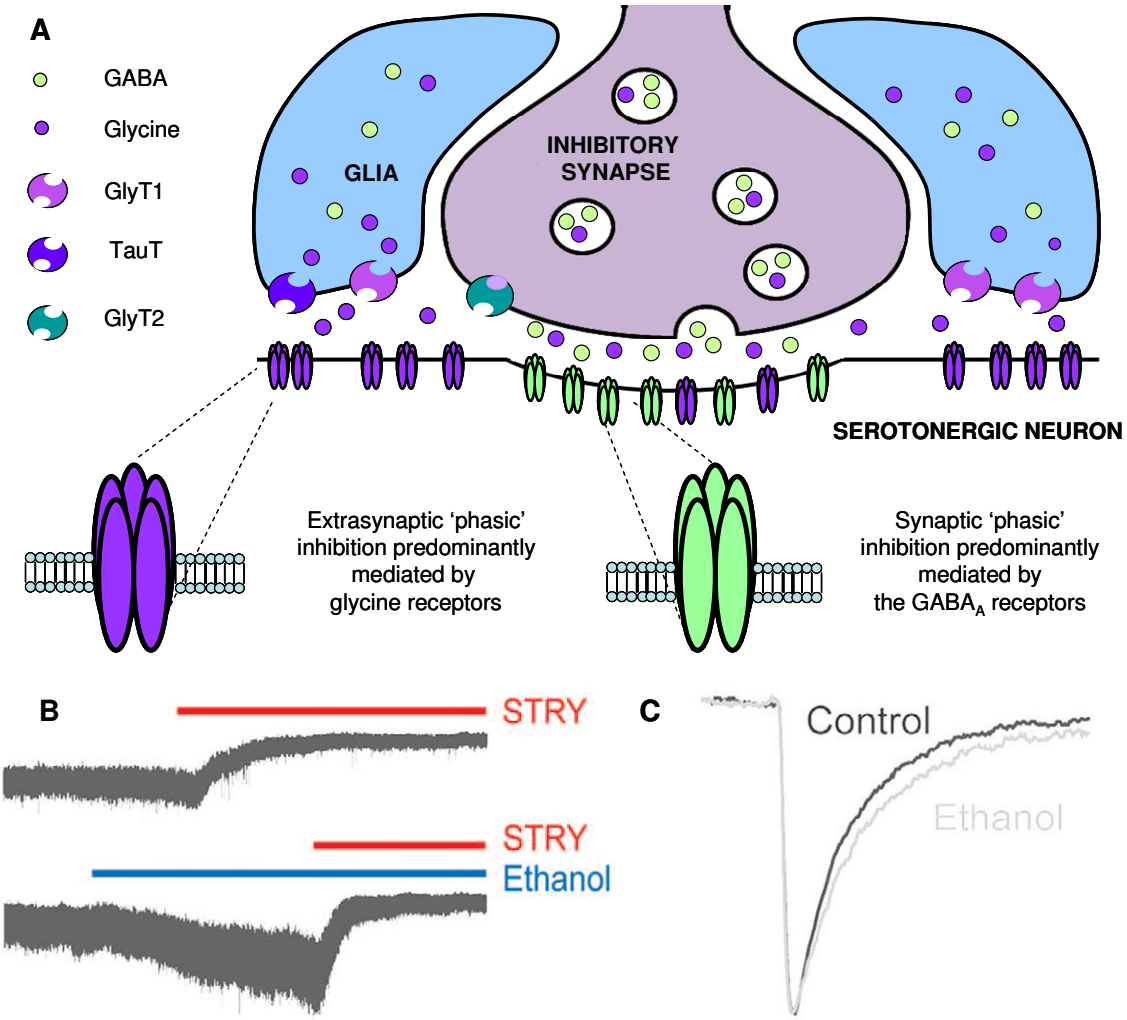

Figure 1.6. – The inhibitory ionotropic GABA$_A$ and glycine receptors which inhibit the neuronal excitation of serotonergic neurons originating in the DRN. Maguire *et al*. (2013) identified that the majority of minature inhibitory post-synaptic currents were bicuculline-sensitive, suggesting the expression of the ionotropic GABA$_A$ receptor (**panel A**). However, the application of bicuculline did not abolish synaptic inhibition. The application of the glycine receptor antagonist strychnine abolished the remainder of the synaptic inhibition identifying the expression of a small synaptic population of strychnine-sensitive glycine receptors. In addition, the application of strychnine induced a large outward shift in the baseline holding current which suggested the expression of a population of tonically active extrasynaptic glycine receptors (**panel B**). The application of low physiological concentrations of ethanol (30 mM) potentiated both the extrasynaptic (**panel B**) and synaptic (**panel C**) glycine-receptor mediated inhibition.

bicuculline, indicating the expression of synaptic $GABA_A$ receptors (Maguire *et al*, 2013; figure 1.6.). In addition, the application of strychnine elicited an outward shift in the baseline holding current suggesting the expression of a population of extrasynaptic strychnine-sensitive glycine receptors (Maguire *et al*, 2013; Farrant and Nusser, 2005; *figure 1.6.*). In agreement, immunohistochemical analysis demonstrated that the majority of strychnine-sensitive glycine receptors did not co-localise with the inhibitory synaptic marker neuroligin2 (Maguire *et al*, 2013). In the presence of phenylephrine, which mimicked the excitatory innervations from the locus coeruleus, the application of glycine mediated a transient, strychnine-sensitive inhibition of neuronal excitation (Maguire *et al*, 2013).

1.5. Strychnine-sensitive glycine receptors

Historically, the strychnine-sensitive glycine receptor was originally purified and cloned from the rat spinal cord and was found to share significant homology with the nicotinic acetylcholine receptor (Pfeiffer *et al*, 1982; Grenningloh *et al*, 1987). Therefore the strychnine-sensitive glycine receptor was identified as a member of the Cys-loop ligand gated ion channel family which includes the γ-aminobutyric acid type A receptor ($GABA_A$), the 5-hydroxytryptamine type 3 (5-HT_3) receptor, the nicotinic acetylcholine receptor (nACh) and the zinc-activated channel.

Investigation of the homologous nicotinic acetylcholine receptor suggests the strychnine-sensitive glycine receptor consists of a pseudosymmetric pentameric arrangement of subunits around a central anion (Cl^-)-conducting pore (Unwin, 2003; Miyazawa *et al*, 2003). Each subunit consists of a large extracellular N-terminal ligand binding domain, four transmembrane domains (TM1-TM4) a

short intracellular segment connecting TM1 and TM2, a short extracellular loop connecting TM2 and TM3, a large intracellular loop connecting TM3 and TM4 and a short extracellular C-terminal domain (*figure 1.7.*).

The structure of the ligand binding domain has been inferred from the ACh-binding protein, a homologous protein derived from the freshwater snail *Lymnea stagnalis* (Brejc *et al*, 2001; Celie *et al*, 2004). The binding site is situated at the interface of the subunits and is comprised of three β-sheets from one subunit and the three β-sheets of the adjacent subunit. The activation of the receptor by glycine induces a conformational change, facilitating the movement of Cl^- ions through the channel pore resulting in the hyperpolarisation of the membrane. The antagonist strychnine, however, binds to a distinct but overlapping binding site (O'Connor *et al*, 1996).

1.5.1. The subunit composition of the strychnine-sensitive glycine receptors

The subunit composition of the strychnine-sensitive glycine receptor exhibits significant diversity (Hernandes and Troncone, 2009). Functional receptors can be comprised solely of the α subunit (homomeric receptors) or may be comprised of both α and β subunits (heteromeric receptors; Lynch, 2009). The recombinant expression of the β subunit in the *Xenopus laevis* oocyte does not form functional receptors, suggesting the co-expression of the α subunit is necessary to form the functional receptor (Grenningloh *et al*, 1990a). The subunit arrangement of the heteromeric receptor remains contentious. Early investigations have suggested a 3α:2β arrangement (Langosch *et al*, 1988), however, a subsequent study by Grudzinska *et al*. (2005) suggested a 2α:3β arrangement of the subunits.

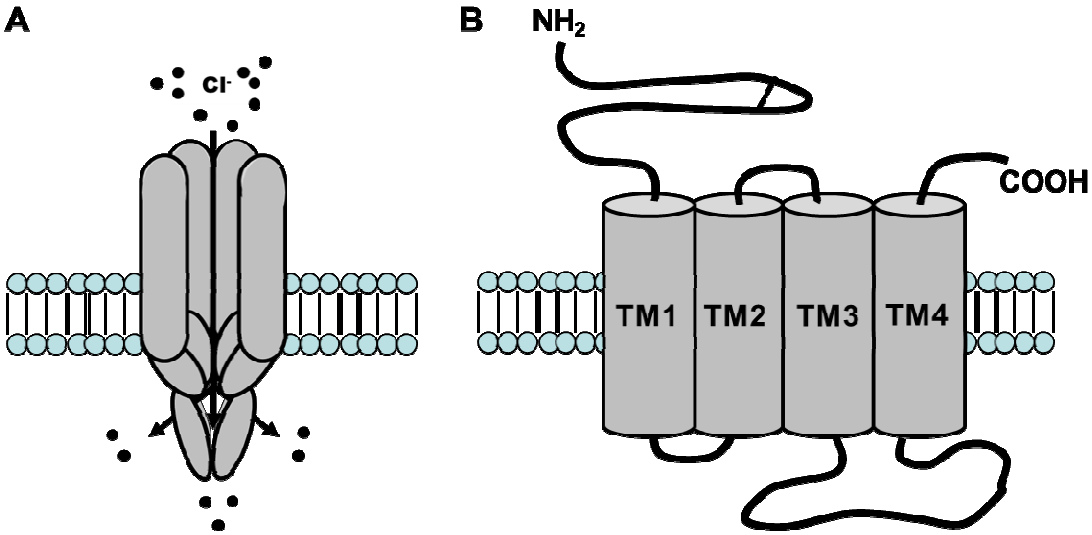

Figure 1.7. – The proposed structure of the strychnine-sensitive glycine receptor and associated subunits. The structure of the strychnine sensitive glycine receptor, minus a single subunit to show the ion conducting channel, is shown in **panel A**. The receptor is comprised of five subunits arranged in a circular fashion to form the central chloride conducting channel. The extracellular domain contains the glycine binding site which is situated at the junction between adjacent subunits. Each subunit consists of four transmembrane domains and includes an extracellular N-terminal domain and a large intracellular loop (**panel B**). The latter of which interacts with intracellular signalling molecules to modulate receptor function. Figures derived from the models of Unwin (2003) and Miyazawa *et al* (2003).

To date, four distinct α subunits (1-4) and one β subunit have been identified, which underpin the functional diversity of the receptor populations (Hernandes and Troncone, 2009). The incorporation of the β subunit in the heteromeric receptor reduces the conductance of the glycine receptor (Lynch, 2009). In addition, the incorporation of the β subunit has been proposed to mediate the location of the receptor. The β subunit co-localises with the scaffolding protein gephyrin, which serves to anchor the receptor to the post-synaptic density (Pfeiffer *et al*, 1982; Schmitt *et al*, 1987). However, single-quantum tracking suggests that the α1β receptor can migrate between synaptic, perisynaptic and extrasynaptic sites (Dahan *et al*, 2003; Muller *et al*, 2008).

The potential functional diversity of the receptors is further increased by reports of post-transcriptional modifications. For example, splice variants are reported for the α1 subunit ($\alpha1^{ins}$ variant) the α2 subunit (α2A and α2B variants; Miller *et al*, 2004) and α3 subunit (α3L and α3K; Nikolic *et al*, 1998). Similarly, Legendre *et al*. (2009) reported RNA editing of the glycine receptor results in high affinity glycine receptors due to a proline to leucine point mutation (α2 P192L and α3 P185L). RNA editing of GlyRs may have specific physiological/pathophysiological significance since a recent report indicates high affinity glycine receptors in the hippocampus may contribute to the pathology of temporal lobe epilepsy (Eichler *et al*, 2009).

1.5.2. The physiological role of the strychnine-sensitive glycine receptor

In common with the homologous $GABA_A$ receptor, the glycine receptor mediates inhibitory neurotransmission in the developed CNS and is highly expressed in the spinal cord, retina, brain stem and caudal brain regions (Frostholm and Rotter, 1985). However, the behavioural role of the strychnine-sensitive glycine receptor has received limited investigation relative to other members of the cys-loop ligand gated ion channel superfamily. However, mutations of the strychnine-sensitive glycine receptor have been identified in the pathology of human startle syndrome (Legendre, 2001).

The cloning of the receptor subunits has demonstrated the high degree of homology between the α subunits (> 90%; Grenningloh *et al*, 1990b) and to a lesser extent the β subunit (47%; Greninngloh *et al*, 1990a). Therefore, the composition and physiological role of native receptors has proved difficult due to the lack of pharmacological agents with the specificity to discriminate between the subunits (Lynch, 2009).

The strychnine-sensitive glycine receptor plays an important role in the mediation of neurodevelopment (Aguayo *et al*, 2004). During the embryonic and juvenile development, the intracellular concentration of Cl^- is high (*see below*) and therefore the activation of the strychnine-sensitive glycine receptors and $GABA_A$ receptors mediate an efflux of Cl^- mediating neuronal depolarization and excitation (Ito and Cherubini, 1991; Hernandes and Troncone, 2009). In addition, the depolarisation induced by the activation of these receptors mediates an influx of Ca^{2+} via voltage-gated Ca^{2+} channels which is thought to regulate downstream gene expression (Hernandes and Troncone, 2009). The α2 subunit is predominantly expressed and is thought to be an important mediator of synaptogenesis (Hernandes and Troncone, 2009). However, during post-natal development the relative expression of the α1 and β subunits increases with a concurrent decrease in the expression of the α2 subunit expression (Malosio *et al*, 1991). This subunit transition coincides with the increased expression of the K^+-Cl^- co-transporter (KCC2) and a decrease of the Na^+-K^+-$2Cl^-$ co-transporter (NKCC1) which collectively regulate the intracellular chloride concentration (Blaesse *et al*, 2009). This developmental switch is thought to thought to be completed by postnatal day 14 (Malosio *et al*, 1991).

1.5.3. The role of transporters in the mediation of the strychnine-sensitive glycine receptor

The uptake of neurotransmitters in the CNS is mediated by a super-family of transporters which include the Na^+ and Cl^- transporters, the Na^+ and K^+ dependent transporters, the vesicular transporters and other amino acid transporters (Richerson and Wu, 2003; Jursky *et al*, 1994). The Na^+ and Cl^- dependent transporters encompass the the amino acid transporters, the GABA transporters, the monoamine transporters and the orphan transporters (Jursky

et al, 1994). The transporter is comprised of twelve transmembrane helices with six extracellular and five intracellular loops, with short intracellular N- and C-terminals and a large extracellular loop located between helices VII and VIII (Jursky *et al*, 1994). The concentration of glycine in the extracellular fluid is mediated by the GLYT1 and GLYT2 Na^+/Cl^- -dependent transporters which are members of the amino acid transporter subdivision (Guastella *et al*, 1992; Liu *et al*, 1993). The GlyT1 and GlyT2 transporters share 50% homology. Five GlyT1 isoforms (a-f) have been identified as well as the GlyT2a and GlyT2b variants (Hernandes and Troncone, 2009).

However there are notable differences between the GlyT1 and GlyT2 transporters. For example, the GlyT1 and GlyT2 transporters differ in their stoichiometry. The GlyT1 transporter requires two Na^+ and one Cl^- to co-transport one glycine molecule. However, the GlyT2 transporter requires three Na^+ and one Cl^- to co-transport one glycine molecule (Roux and Supplisson, 2000). In addition, the two transporter subtypes demonstrate distinct neuronal localisations. GLYT1 is expressed by glial cells, whereas GLYT2 is expressed exclusively by neurons (Jursky *et al*, 1994). However, these high-affinity transporters may serve a dual role in the mediation of the concentration of glycine in the extracellular space. In addition to uptake, these transporters may be reversed to and therefore mediate a paracrine release of glycine (Richerson and Wu, 2003). The reversal of the Na^+/Cl^- dependent transporters GlyT1 and GlyT2 may mediate this paracrine release in response to a depletion of the Na^+ gradient (Legendre, 2001). The localisation of GlyT1 on glial cells, in contrast to the neuronal GlyT2, implies that GlyT1 may predominate in this release process (Legendre, 2001).

The GlyT1 and GlyT2 proteins show distinct regionally-selective expression in the CNS. The GlyT1 transporter is expressed ubiquitously throughout the CNS (Adams *et al.*, 1995; Jursky *et al.*, 1994). Although predominantly expressed on astrocytes, the GlyT1 transporter is expressed on glutamatergic neurons and regions of the brain abundant in NMDA receptor expression such as the neocortex and hippocampus (Cubelos *et al.*, 2005). Conversely, the GlyT2 transporter is localised to regions which are abundant in glycinergic innervation and express the strychnine-sensitive glycine receptor such as the spinal cord and brain stem (Jursky *et al*, 1994; Rampon *et al.*, 1996).

In addition, the transporter for taurine (*section 1.5.4.*) is member of the GABA transporter family. Similar to the GlyT transporter, the TauT transporter exists as both the TauT1 and TauT2 isoforms which are largely homologous (> 60%). The TauT1 transporter is predominantly expressed in retinal neurons and the cerebellum, whereas the mRNA encoding the TauT2 isoform has been identified in the brain stem, striatum, cerebellum and corpus callosum (Vinnakota *et al.*, 1997).

1.5.4. Agonists of the strychnine-sensitive glycine receptor

The amino acid glycine was originally proposed as a neurotransmitter based on its association with interneurons in the spinal cord (Davidoff *et al*, 1967). The electrical stimulation of slices of the rat spinal cord evoked the release of ^{14}C-glycine (Hopkin and Neal, 1970). Subsequent experiments showed that the electrophoretic administration of glycine evoked a strychnine-sensitive inhibitory current in motorneurons (Curtis *et al*, 1967). When recombinantly expressed in the Xenopus oocyte, the homomeric α1 strychnine-sensitive glycine receptor was shown to be activated by glycine (Schmieden *et al*, 1992). In addition to

glycine, β-alanine and taurine have been identified as endogenous agonists of the glycine receptor. Of these endogenous agonists, glycine is the most potent and taurine the least efficacious (reviewed by Lynch, 2009). Site directed mutagenesis studies have indentified the amino acids in the N-terminal domain (R65 and E157 of the alpha1 subunit and R86 and E180 of the beta subunit) which form the glycine binding pocket at the interface between subunits of the strychnine-sensitive glycine receptor (Grudzinska *et al*, 2005).

Similarly, the amino acids 111 and 212 (Schmieden *et al*, 1992) and 58 and 59 (Miller *et al*, 2004) were identified as critical determinants of the binding of β-alanine and taurine respectively.

1.5.5. Antagonists of the strychnine-sensitive glycine receptor

Strychnine is a natural alkaloid extract from the seeds of the *Strychnos Nux-vonica* and is commonly used as a pesticide for small vertebrates (Makarovsky *et al*, 2008). Intoxication causes convulsions and muscle spasms which result in respiratory failure. The toxic effects of strychnine are attributed to the high affinity of strychnine for the glycine receptors expressed in the spinal cord (Young and Snyder, 1973). The presence of strychnine binding to the inhibitory glycine receptor abolishes the activation of the receptors in response to application of the endogenous agonist glycine (Houamed *et al*, 1984).

The binding site of strychnine is distinct but overlaps with the glycine binding site to mediate the antagonistic effect. Site-directed mutagenesis studies have implicated amino acids 157 and 131 as critical determinants of strychnine binding (O'Connor *et al*, 1996). In addition, investigations of the heteromeric αβ glycine receptor implicated the 180 residue in the binding of strychnine (Grudzinska *et al*, 2005). Although strychnine demonstrates a high affinity for all

strychnine-sensitive glycine receptors, it exhibits a higher affinity for the heteromeric receptor as opposed to the homomeric receptor (Pribilla *et al*, 1992). Due to this specificity, strychnine remains an important tool in the study of strychnine-sensitive glycine receptors. However, it should be noted that at higher concentrations strychnine has also been reported to antagonise $GABA_A$ receptor function (Bowery and Smart, 2006).

Picrotoxin, in addition to act as non-competitive antagonist of the $GABA_A$ receptor, blocks strychnine-sensitive glycine receptors although it exhibits a differential effect at homomeric vs. heteromeric receptors (Pribilla *et al*, 1992; Laube *et al*, 2002). This may be due to the relative affinities for the receptor of the two molecules which comprise picrotoxin – namely picrotin and picrotoxinin (Yang *et al*, 2007). Unlike strychnine, picrotoxin is a non-competitive antagonist which is thought to block the central ion conducting channel of the receptor through interactions with the pore lining residues.

Additional antagonists of the strychnine-sensitive glycine receptors include cyanotriphenylborate (Rundstrom *et al*, 1994), a non-competitive open-channel blocker, and the $GABA_A$ receptor antagonists bicuculline and gabazine which competitively antagonise the receptor at higher concentrations than those that inhibit the $GABA_A$ receptor (Wang and Slaughter, 2005; Beato *et al*, 2007).

1.5.6. Allosteric modulators of the strychnine-sensitive glycine receptor

Although few compounds specifically act upon the strychnine-sensitive glycine receptor, however, a diverse range of molecules have been reported to modulate glycine receptor function albeit not exclusively. These include different cations such as zinc, cannabinoids, neuroactive steroids, tropeines, alcohols,

avermectins, butyrolactones and general anaesthetics (Yevenes and Zeilhofer, 2011).

Endocannabinoids and related molecules have been reported to modulate the strychnine-sensitive glycine receptors. In hippocampal neurons, the endogenous cannabinoids anandamide (AEA) and 2-arachidonyl-glycerol (2-AG) reduced the amplitude of glycinergic mIPSCs in addition to the rise time, desensitisation and deactivation kinetics in a concentration-dependent manner (Lozovaya *et al*, 2005). These effects were insensitive to CB1 antagonists and remained despite the administration of the G protein inhibitor GDP-β-S (Lozovaya *et al*, 2005). In addition, potentiation of the recombinant expression of the α1 homomeric glycine receptor was reported in *Xenopus laevis* oocytes in response to AEA in a manner independent of the CB1 receptor (Hejazi *et al*, 2006). Xiong *et al*. (2011) have implicated a serine residue in the TM3 region (S296 in α1 and S307 in α3) in the potentiating effects of Δ9-tetrahydrocannabinol (the active ingredient of the *Cannabis sativa* plant) on the α1 and α3 glycine receptors.

Volatile anaesthetics such as isoflurane, enflurane, halothane and sevoflurane potentiate the inhibitory currents of recombinantly expressed homomeric α1 glycine receptors in response to the administration of glycine (Downie *et al*, 1996; Belelli *et al*, 1999; Krasowski and Harrison, 1999; Mascia *et al*, 1996). In addition, homomeric α2 glycine receptors are sensitive to isoflurane (Harrison *et al*, 1993). Due to the expression of glycine receptors in the spinal cord, the effects modulation of the glycine receptors has been proposed to mediate the immobility associated with anaesthesia by such agents (Rudolph and Antkowiak, 2004). The volatile anaesthetics have been proposed to act at a binding pocket comprised of the TM2 and TM3 domains in the α1 homomeric

receptor (Mihic *et al*, 1997). Amongst the intravenous general anaesthetics, propofol potentiates the activity of the strychnine-sensitive glycine receptor albeit at concentrations significantly higher than those acting upon $GABA_A$Rs (Hales and Lambert, 1991; Pistis *et al*, 1997a).

The avermectins are commonly used as anti-parasitic and insecticide agents and are derived from the *Streptomyces avermitilis* bacterium (Yevenes and Zeilhofer, 2011). Ivermectin, a member of the avermectins, has been shown to activate recombinant α1 homomeric and α1β heteromeric glycine receptors (Shan *et al*, 2001) with the A288 residue residing in the TM3 domain of the α1 homomeric receptor being specifically implicated in such actions (Lynagh and Lynch, 2010).

The endogenous neurosteroids are cholesterol based metabolites synthesised in the CNS. The 3α reduced neurosteroids include 5α-pregnan-3α-ol-20-one and 5β-pregnan-3α-ol-20-one and have been shown to facilitate inhibition mediated by the $GABA_A$ receptor. However, these neurosteroids have negligible effects on the strychnine-sensitive glycine receptor (Lambert *et al*, 2001; Pistis *et al*, 1997a; Weir *et al*, 2004). In contrast, a number of synthetically derived neurosteroids such as minaxolone, Org20599 and alphaxalone have been shown to potentiate the strychnine-sensitive glycine receptor (Ahrens *et al*, 2008; Weir *et al*, 2004). However, this potentiation occurred at concentrations higher than those reported at the $GABA_A$ receptor (Weir *et al*, 2004).

The tropeines are potent 5-HT_3 antagonists and are most commonly used in conjucture with chemotherapy as an antiemetic (Yevenes and Zeilhofer, 2011). In cultured spinal neurons, the tropeines MDL-72222 and tropisetron potentiate glycine receptor-mediated currents at low nanomolar concentrations (Chesnoy-

Marchais, 1996). However, at higher micromolar concentrations caused an inhibition. In addition, tropisetron potentiates α1 but inhibits α2 homomeric receptors and therefore demonstrating subunit-specificity in recombinant expression systems (Supplisson and Chesnoy-Marchais, 2000). In addition, the incorporation of the β subunit switched inhibition to potentiation in α2 containing receptors. Site-directed mutagenesis studies showed that mutation of the α1 (N102) but not the β subunit (N125) abolished tropisetron mediated inhibition without altering potentiation. Therefore, the potentiating and inhibitory effects of tropisetron may be mediated by distinct sites on the strychnine-sensitive glycine receptor.

The best characterised allosteric modulator of the strychnine-sensitive glycine receptor is the cation zinc. Zinc modulates the strychnine-sensitive glycine receptor in a bi-phasic manner. At low doses (< 10 μM) potentiation predominates and is mediated by increasing the receptor affinity for glycine. In contrast, at higher doses zinc exerts an inhibition of the strychnine-sensitive glycine receptor (Bloomenthal *et al*, 1994; Laube *et al*, 2000). These bi-directional effects are mediated at two distinct binding sites. The residues forming the binding site for the positive allosteric modulation have been identified as D80, E192 and E194 (Laube *et al*, 2000; Lynch *et al*, 1998). In contrast, H107, H109, T112 and T133 in the α1 glycine receptor have been implicated as residues critical to the binding site responsible for the inhibitory effect (Harvey *et al*, 1999; Laube *et al*, 2000; Miller *et al*, 2005). In addition, the α1 homomeric glycine receptor shows increased sensitivity to the inhibitory effects of zinc than the α2 and α3 homomeric glycine receptors. The homozygous D80 transgenic mouse, a mutation shown to reduce the

potentiating effects of zinc in recombinant systems, demonstrated a progressive hyperekplexia-like phenotype (Yevenes and Zeilhofer, 2011).

In addition, previous studies have identified interactions between ethanol and the function of the strychnine-sensitive glycine receptor. As this will form a significant basis for the present study, the physiological effects of ethanol and interactions with specific neurotransmitter systems and direct cellular targets will be discussed in depth (*section 1.6.1. and 1.6.2. respectively*).

1.6. Ethanol

Ethanol, the active substance of alcoholic beverages, is the most commonly used drug in the world due to its legal status, cultural significance and availability. Using a multicriteria decision analysis, Nutt *et al*. (2010) suggested that alcohol is the most harmful drug used in the UK based on factors influencing the cost to the individual and society in general. At low doses, the consumption of ethanol can induce the loss of fine motor control. However, at intermediate to high doses ethanol can elicit sedation, anxiolysis and anticonvulsant actions and anaesthesia (Little, 1991). The excessive consumption of ethanol can suppress respiratory function which, if untreated, can be fatal (Eckardt *et al*, 1998). Due to the dose-dependent nature of ethanol's effects on the CNS, several definitions have been proposed as to what constitutes "moderate" ethanol consumption (Eckardt *et al*, 1998). However, the investigation of the effects of ethanol in humans is difficult due to the array of factors associated with the peripheral organs which determine the concentration of ethanol present in the CNS – the rate of absorption from the gastrointestinal tract, the volume of distribution of the body and the rate of metabolism.

In the fasted state, ethanol transitions through the empty stomach rapidly and is predominantly absorbed through the duodenum and jejunum. However, solid foods and hypertonic solutions slow gastric emptying and therefore ethanol is predominantly absorbed through the stomach which occurs at a slower rate than in the duodenum and jejunum (Cortot *et al*, 1986). Therefore, the site at which ethanol is primarily absorbed influences the concentration of ethanol entering the systemic circulation. In addition, the rate of ethanol absorption influences the rate of first pass metabolism. A reduced rate of ethanol absorption is associated with greater first pass metabolism (Eckardt *et al*, 1998). The slowing of gastric emptying reduces the concentration of ethanol in the portal circulation, therefore, reducing the concentration exposed to the liver. In turn, this reduces the concentration of ethanol which enters the systemic circulation.

In addition, the distribution of ethanol within the body varies with the vascularisation of the tissue due to the fact that ethanol distributes itself in total body water. At equilibrium, the distribution of ethanol between the intracellular and extracellular compartments is approximately equal. However, the establishment of this equilibrium is quicker in well vascularised tissues such as the brain, liver, lung and kidneys (Eckardt *et al*, 1998).

Ethanol metabolism in the liver is predominantly mediated by alcohol dehydrogenase (ADH) enzymes, which converts ethanol to the metabolite acetaldehyde and is the rate-limiting step in ethanol metabolism. The ADH peptide is dimeric and contains two atoms of zinc per subunit. In humans, seven ADH genes and enzymes (ADH1-ADH7) have been identified and are catagorised in five groups based on their kinetics, electrophoretic mobility and sensitivity to pyrazole derivatives. Acetaldehyde is, in turn, metabolised by

aldehyde dehydrogenase (ALDH) enzymes to form acetate. Similarly, there are numerous isoforms of the tetrameric ALDH in the human liver. However, only the class I (ALDH1) and class II (ALDH2) isozymes are thought to mediate the oxidation of acetaldehyde. Therefore, genetic variation in the enzyme isoforms and their relative expression in the liver account for a component of the inter-individual variation of ethanol's pharmacological effects mediated in the CNS (Eckardt *et al*, 1998). In agreement, a study in monozygotic and dizygotic twins estimated that 62% of inter-individual variation in peak blood alcohol content and 49% of variability in the elimination rate of ethanol was due to genetic factors (Kopun and Propping, 1977). In addition, ethanol elimination rates are influenced by a number of factors including the individual's history of alcohol consumption, smoking, diet, age, sex, weight and the time of day the alcohol is consumed.

1.6.1. The pharmacology of ethanol

Ethanol was originally proposed to alter the chemico-physical properties of neuronal membranes by a non-specific mechanism (McCreery and Hunt, 1978). As an amphipathic molecule, ethanol was hypothesised to perturb the physical properties of the lipid neuronal membrane and therefore alters the function of membrane bound proteins. However, this hypothesis was dismissed by evidence suggesting that the effect of ethanol on the fluidity of the membrane were negligible at physiological relevant concentrations (Eckardt *et al*, 1998). In the last 20 years or so significant progress has been made in identifying specific interactions between ethanol and endogenous neurotransmitter systems. In particular, investigations have focussed on the dopaminergic, GABAergic, glutamatergic and serotonergic and opioid systems in addition to the intracellular signalling pathways of the CNS. In more recent years, the inhibitory

glycinergic system has also gained significant weight in the mechanism of ethanol action as discussed in detail section (*section 1.6.2.5.*). As mentioned previously, the behavioural effects of ethanol are highly temporal and dose-dependent and therefore the effects are mediated by the summation of these component interactions. Therefore, the effects of ethanol on the neurotransmitter systems associated with dependence will be discussed initially followed by a subsequent review of the evidence supporting direct interactions with cellular proteins.

The mesolimbic dopamine system has been extensively investigated in the role of mediating the reinforcing effects of many substances including ethanol (Koob, 1992). In vivo recordings have demonstrated that the administration of low doses of ethanol induce an increase in the firing rate of mesolimbic dopaminergic neurons (Mereu *et al*, 1984; Gessa *et al*, 1985). In agreement, dopamine overflow in the nucleus accumbens is increased by the systemic administration of ethanol in rats (Imperato and Di Chiara, 1986). In addition, an ethanol induced increase in dopamine overflow in the caudate nucleus has been reported. However, this effect was induced by doses of ethanol higher then those reported to increase dopamine overflow in the nucleus accumbens (Imperato and Di Chiara, 1986).

Previous studies have identified that the endogenous opioid system plays an important role in the reinforcing effects of ethanol (Froehlich and Li, 1994). In particular the opioid receptors mediate, in part, the ethanol induced stimulation of dopamine release. Using microdialysis, the systemic administration of naltrexone (at doses which blocks μ and δ opioid receptors) reversed the stimulation of dopamine overflow in the rat nucleus accumbens induced by locally applied ethanol (Benjamin *et al*, 1993). In addition, Acquas *et al*. (1993)

demonstrated that the local administration of the δ receptor antagonist naltrindole suppressed the release of dopamine evoked by the systemic administration of ethanol. Recently, the Scottish Medicines Consortium has approved the use of the μ opioid receptor antagonist nalmefene, a homologue of naltrexone, in the treatment of alcohol dependence (http://www.bbc.co.uk/news/uk-scotland-24431152; accessed 15/10/13).

In addition, there is significant evidence supporting an interaction between the pharmacological effects of ethanol and the serotonergic system, however, this will be addressed in depth in section 1.6.3.

1.6.2. The interactions between ethanol and cellular targets

1.6.2.1. The $GABA_A$ Receptor

The specific interaction of ethanol and the $GABA_A$ receptor was historically based upon the common behavioural outcomes of ethanol and $GABA_A$ receptor allosteric modulators, such as the benzodiazepines, which include anxiolysis, sedation, anticonvulsive and anaesthesia (Weiner and Valenzuela, 2006). In agreement, electrophysiological evidence from cultured hippocampal and cortical neurones suggest that ethanol is a positive allosteric modulator of the $GABA_A$ receptor at concentrations as low as 1 mM (Aguayo *et al*, 1990; Reynolds and Prasad, 1991). In addition, in vivo studies have shown that the systemic administration of ethanol potentiates the inhibitory effect of GABA on neuronal activity in the inferior collicular cortex (Simson *et al*, 1991a). However, the potentiating effects of ethanol are not ubiquitous throughout the CNS (Osmanovic and Shefner, 1990). Therefore, the potentiating effects of ethanol were thought to be mediated by subunit specific interactions which would account for the regionally specific effects reported. In agreement, Wafford *et al*.

(1991; 1992) identified that the long form of the γ_2 subunit (γ_{2L}) was necessary for the potentiation of the $GABA_A$ receptor by ethanol. The γ_{2L} subunit contains an additional 8 amino acid sequence which mediated a protein kinase C phosphorylation site. In agreement, mutant mice lacking the brain-specific γ isoform of PKC show a reduced sensitivity to ethanol in vivo (Harris *et al*, 1995). More recent studies have focussed on the δ subunit-containing $GABA_A$ receptor which is expressed at extrasynaptic sites (Farrant and Nusser, 2005) and has been reported to be potentiated by low physiologically relevant concentrations of ethanol although this proposal remains highly controversial (Korpi *et al*, 2007).

1.6.2.2. The NMDA receptor

Electrophysiological evidence suggests ethanol exerts an inhibitory effect on the excitatory NMDA receptor. Lovinger *et al*. (1989) demonstrated that the excitatory current evoked by the application of NMDA in voltage-clamped hippocampal cells was inhibited by ethanol in a concentration dependent manner. In agreement, the application of ethanol to cerebellar granule neurons reduced NMDA-mediated $^{45}Ca^{2+}$ uptake and cGMP production (Hoffman *et al*, 1989). The i.p. administration of ethanol inhibits the NMDA mediated neuronal activity in the rat medial septum (Simson *et al*, 1991b). Similar to the $GABA_A$ receptor, the effects of ethanol on the NMDA receptor are regionally-selective. However, subunit specificity for the NMDA receptor is not well defined. Generally, studies have reported that the GluN1/N2A and GluN1/N2B are slightly more sensitive to ethanol than GluN1 combined with GluN2C or GluN2D (Kuner *et al*, 1993; Möykkynen and Korpi, 2012). Similarly, the AMPA and kainate receptors have been reported to be inhibited by ethanol (Lovinger, 1993; Wirkner *et al*, 2000; Läck *et al*, 2008). However, the mechanism of

ethanol mediated inhibition of the glutamate receptors remains to be determined (Möykkynen and Korpi, 2012).

1.6.2.3. The nicotinic acetylcholine receptor

An investigation of ethanol actions upon the nicotinic acetylcholine receptor was first proposed on the basis of the observed interaction between consumption of ethanol and cigarette smoking. In a study of moderate social drinkers (defined as 4-10 drinks/week), Mitchell *et al*. (1995) reported an association between increased cigarette smoking in the ascending limb of the blood alcohol curve. In mice, ethanol induced stimulation of dopamine overflow in the nucleus accumbens and locomotor activity were reduced by the administration of the nACh receptor antagonist mecamylamine (Blomqvist *et al*, 1996). In addition, mecamylamine has been shown to reduce voluntary ethanol intake in alcohol-preferring rats (Blomqvist *et al*, 1996).

1.6.2.4. The 5-HT$_3$ receptor

Low to moderate concentrations of ethanol have negligible effects on either the 5-HT$_{1A}$ or 5-HT$_2$ receptors (Eckardt *et al*, 1998). However, ethanol has been reported to interact with the ionotropic 5-HT$_3$ receptor which is predominantly expressed in mesolimbic regions of the brain (Kilpatrick *et al*, 1987). Electrophysiological experiments have reported that ethanol potentiates the serotonin-induced activation of 5-HT$_3$ receptors expressed in neuroblastoma cells and human embryonic kidney cells (Lovinger, 1991a; Lovinger and Zhou, 1994). In addition, this effect was blocked by the addition of the 5-HT$_3$ receptor antagonist tropisetron (ICS 205-930; Lovinger, 1991b). The subcutaneous administration of the 5-HT$_3$ antagonist tropisetron reduced the increase in dopamine overflow in the nucleus accumbens induced by the i.p. administration

of ethanol (Carboni *et al*, 1989). In agreement, the antagonists of the 5-HT_3 receptor have been reported to reduce voluntary ethanol intake in alcohol-preferring rats (Fadda *et al*, 1991; Kostowski *et al*, 1993). Therefore, these studies suggest an interaction between the dopaminergic and serotonergic systems contributes to the behavioural effects of ethanol.

1.6.2.5. The strychnine-sensitive glycine receptor

A number of recent studies have reported that the inhibitory glycinergic system plays a role in mediating the effects of ethanol. In Long-Evans rats, the administration of glycine into the VTA was found to reduce ethanol consumption in a strychnine-dependent manner (Li *et al*, 2012). In the nucleus accumbens (nAc), the administration of strychnine abolishes the ethanol induced increase in dopamine overflow (Molander and Soderpalm, 2005). In agreement, the intra-nAc administration of glycine (100 µM by reverse dialysis) increased dopamine overflow in a subpopulation of ethanol preferring Wistar rats which reduced ethanol preference and intake (Molander *et al*, 2005). The acamprosate-induced reduction of ethanol intake was found to be reversed by intra-nAc administration of strychnine (Chau *et al*, 2010). Collectively, this suggests that the strychnine-sensitive glycine receptors play a role in the interaction between ethanol and the dopaminergic system.

In agreement with the studies described above, evidence from electrophysiological experiments consistently implicates ethanol as a positive allosteric modulator of the strychnine-sensitive glycine receptor at physiological concentrations thought to reflect mild intoxication. This potentiation originates from a decrease in the EC_{50} without a change in maximal currents (Aguayo et al, 1996; Mihic, 1999). Potentiation of the strychnine-sensitive glycine receptor

has been reported in the spinal cord, hippocampus, hypoglossal nucleus and ventral tegmental area (Aguayo *et al*, 1996; Eggers and Berger, 2004; Jiang and Ye, 2003). At concentrations below 100 mM, the homomeric α1 receptor is more sensitive to ethanol than the α2 receptor (Mascia *et al*, 1996; Perkins *et al*, 2008; Yevenes *et al*, 2010). Site-directed mutagenesis studies have reported that S267 of the TM2 region and A288 of the TM3 region, thought to form a water filled cavity, abolish the potentiating effects of ethanol in the α1 homomeric receptor (Mihic *et al*, 1997; Mascia *et al*, 2000; Ye *et al*, 1998). In addition, the ethanol modulation of the strychnine-sensitive glycine receptor may be mediated by an indirect action via a intracellular signalling pathways. For example, ethanol mediated potentiation of the native and recombinant strychnine-sensitive glycine receptors is attenuated by PKC inhibitors (Jiang and Ye, 2003; Mascia *et al*, 1998). The generation of the Gβγ-insensitive α1 receptor attenuated the modulatory effects of ethanol; however, there was no effect on the potentiation induced by general anaesthetics (Yevenes *et al*, 2008). Therefore both direct and indirect interactions with the glycine receptor are likely to mediate the positive allosteric modulation of ethanol.

In addition, Maguire *et al*. (2013; *figure 1.6.*) have recently demonstrated that ethanol directly modifies the strychnine-sensitive glycine receptors of the DRN to facilitate the inhibition of the serotonergic neurons. The application of a physiologically relevant concentration of ethanol (30 mM) induced a strychnine-sensitive inward current (Maguire *et al*, 2013; *figure 1.6.*). Similarly, the pre-application of glycine and subsequently ethanol induced a strychnine-sensitive inward current suggesting that ethanol potentiated the glycine receptor-mediated inhibition of the serotonergic neurons (Maguire *et al*, 2013). To ensure the effects of ethanol were not due to osmotic pressures, the administration of a

mannitol-based hyper-osmotic solution had no effect on the baseline holding current (Maguire *et al*, 2013). In addition, ethanol had no effect on the frequency or amplitude of the glycine receptor-mediated mIPSC but prolonged the decay time (Maguire *et al*, 2013). Therefore the effects of ethanol are mediated by both the synaptic and extrasynaptic glycine receptors.

1.6.3. The effects of ethanol on the serotonergic system

Clinical evidence suggests that a proportion of alcohol-dependent individuals have lowered central serotonin (LeMarquand *et al*, 1994a). Therefore, Tabakoff and Hoffman (1991) suggested that ethanol facilitates serotonergic transmission and that alcoholics consume alcohol to self-medicate hypoactivity of serotonergic transmission. In agreement, the intra-ventricular administration of 5,6-DHT in Sprague-Dawley rats increased ethanol consumption (Melchior and Myers, 1976). In addition, the majority of studies investigating tissue levels of serotonin and the metabolite 5-HIAA in animals suggest that the acute administration of ethanol decreases serotonin in the brain and increases 5-HIAA suggesting an increase in the turnover of serotonin (LeMarquand *et al*, 1994b). Langen *et al*. (2002) reported that the systemic administration of 1 g/kg of ethanol evoked a significant increase in serotonin overflow in the medial prefrontal cortex of Wistar-Harlan rats. Serotonergic neurons in the medial prefrontal cortex originate from the DRN (Azmitia and Segal, 1978) and therefore the results would support the hypothesis that ethanol facilitates serotonergic transmission.

However, an increase in the turnover of serotonin in the brain is not necessarily due to an increase in the excitation of serotonergic neurons. For example, this may be mediated by an ethanol induced inhibition of the clearance of 5-HIAA

(Nutt and Glue, 1986). Although, such an effect would likely induce a global increase in brain 5-HIAA which is not the case as previous studies suggest the effects of ethanol on 5-HIAA are regionally selective (LeMarquand *et al*, 1994b). In addition, an alternative explanation for the increase in serotonin overflow reported by Langen *et al*. (2002) is that the serotonin transporter is inhibited by the administration of ethanol (Daws *et al*, 2006). However, the concentrations of ethanol used by Daws *et al*. (2006) to inhibit ethanol uptake were higher than those used by Langen *et al*. (2002). In addition, *in vivo* electrophysiological studies have shown that the systemic administration of ethanol in anaesthetised Sprague-Dawley rats induces a dose-dependent suppression of serotonergic neuronal excitation in the DRN (Chu *et al*, 1984; Pistis *et al*, 1997b). In agreement, the study of acute ethanol intake in healthy individuals suggests that ethanol lowers blood tryptophan, tryptophan in the cerebrospinal fluid, blood serotonin and increases platelet serotonin uptake (LeMarquand *et al*, 1994a). Collectively these peripheral measures would suggest a decrease in central serotonin levels.

Collectively, the majority of studies have reported that ethanol facilitates serotonergic transmission. The role of serotonin in the mediation of anxiety has been discussed previously and, in general, the evidence suggests that serotonin facilitates anxiety. Therefore, there is an apparent contradiction. If ethanol facilitates serotonergic transmission then ethanol should promote anxiety and not induce an anxiolytic effect.

1.6.4. The anxiolytic effects of ethanol in humans

The idea that the consumption of alcohol can reduce anxiety or responses to stress is based on reports and experiences that alcohol is associated with an

increased social interaction in humans. Extensive evidence suggests that ethanol and the anxiolytic benzodiazepines both potentiate responses at the $GABA_A$ receptor. Historically, the transition from moderate to excessive or pathological alcohol consumption was thought to be due to the positive affect and tension reducing effect evoked by alcohol and was refered to as the "tension-reduction hypothesis" (Pohorecky, 1981; Pohorecky, 1991). In agreement, most alcoholics report that relief of anxiety is an important reason for drinking (Edwards *et al*, 1972). However, the anxiolytic effect of ethanol is reported in the ascending limb of the blood alcohol concentration curve and is subsequently followed by a more prolonged period of anxiety and depression (Pohorecky, 1981). Studies of the anxiolytic effect of alcohol in humans have been difficult to interpret because of the factors stated previously and the role of expectancy (Eckardt *et al*, 1998). It has been reported that the anticipated effects of ethanol may have a greater influence than the pharmacological effects of ethanol. In addition, Wilson (1988) noted that the nature of the test administered is important due to the fact that ethanol may affect self-rating and autonomic measures of anxiety differently.

Wilson *et al*. (1989) reported that a moderate dose of ethanol elicited a decrease in systolic blood pressure, but had no influence on either heart rate or or diastolic blood pressure in female social drinkers exposed to social stress. Conversely, Lipscomb *et al*. (1980) reported a dose-dependent decrease in heart rate in male social drinkers in response to social stress. Therefore, the effects of ethanol on the autonomic responses to anxiogenic stimuli are inconsistent in the literature. However, ethanol has been reported to reduce self-ratings of anxiety on Spielberger's State Anxiety scale in female social drinkers with positive expectations of ethanol's action, but not in others (De

Boer *et al*, 1994). However, Bond and Silveira (1993) found no effects of ethanol in a stressful competitive task. Similarly, Lister and File (1983) reported no effect of alcohol on students' self-ratings of anxiety as measured on the Bond and Lader's analogue scale but did report a reduction in anxiety on the Spielberger State Anxiety scale during testing under high and low stress conditions.

The "self-medication" hypothesis proposed that the anxiolytic effect of ethanol facilitates the consumption of alcohol and therefore individuals with high anxiety will consume more alcohol than nonanxious individuals. The hypothesis is derived from the association of high alcohol intake in individuals diagnosed with an anxiety disorder. Chutuape and de Wit (1995) tried to address the "self-medication hypothesis" by investigating whether anxiety upon consumption is reduced in individuals with anxiety disorders, and whether a decrease in anxiety is associated with increased preference for alcohol. The study found that alcohol decreased anxiety in the individuals with an anxiety disorder. However, the decrease in anxiety was not associated with an increased preference for alcohol. In addition, alcohol had minimal effects on the anxiety levels of the control group.

In summary, the investigation of the putative anxiolytic effects of ethanol in humans is inconclusive to date. However, this is due to a multitude of factors that are difficult to control for. Therefore, it would be assumed that animal work would offer greater insight due to the increased control of potentially confounding factors and the multitude of behavioural tests which evoke an anxious state.

1.6.5. The anxiolytic effects of ethanol in animals

In general, the concentration range which elicits anxiolytic effects in rats is lower in rats than it is in mice (Eckardt *et al*, 1998). In addition, the anxiolytic effects of ethanol reported in mice often occur in conjunction with a stimulation of locomotor activity and the two effects are difficult to dissociate experimentally (Eckardt *et al*, 1998). Conversely, the investigation of the anxiolytic effect of ethanol in rats is limited by the sedative and ataxic effects of ethanol at higher doses (Eckardt *et al*, 1998). In agreement, File (1980) reported that the systemic administration of 0.4 and 0.8 g/kg ethanol (i.p.) elicited anxiolytic effects in the social interaction test. However, the administration of 1.2 g/kg produced sedatory effects. Similarly, anxiolytic effects have been reported in the elevated plus maze following the administration of doses of ethanol ranging from 0.5 – 0.75 g/kg (i.p.; Criswell *et al*, 1994). Criswell *et al*. (1994) reported that the sedative component of ethanol's effects were evident at 1 g/kg in the elevated plus-maze. Blanchard *et al*. (1990a) reported that the i.p. administration of 1.2 g/kg of ethanol increased transitions between the front and back of the cage and increased locomotor activity in response to exposure to a predator stressor characteristic of an anxiolytic effect. In the Geller-Seifter conflict task, 0.75 g/kg (i.p.) suppressed response inhibition but had no effect on unpunished responses (Koob *et al*, 1988). Similarly, doses of ethanol ranging from 0.5 – 2 g/kg were reported to be anxiolytic in a punished drinking conflict task; however, higher doses elicited sedative effects (Glowa *et al*, 1988).

Attempts have been made to reverse the anxiolytic effects of ethanol. The administration of RO 15-4513, a partial agonist of the benzodiazepine binding site, reversed the anxiolytic-like effect of ethanol in the elevated plus-maze. However, RO 15-4513 reduced the total number of arm entries when given alone (Prunell *et al*, 1994b). In agreement, the administration of pregnenolone

and pregnenolone sulphate in mice reversed the anxiolytic effects of ethanol in the elevated plus-maze, however, the doses were anxiogenic when administered alone (Melchior and Ritzmann, 1994).

In an attempt to address the “self-medication hypothesis”, the anxiety-like behaviours of animals which show a high preference for alcohol have been investigated. Stewart *et al*. (1993) reported that the alcohol preferring P line of rats had a higher level of anxiety in the elevated plus-maze and Geller-Seifter conflict task than the NP line. In agreement, similar results were reported for the Sardinian alcohol preferring line when compared to the non-preferring strain (Colombo *et al*, 1995). However, the alcohol-preferring AA line did not differ from the non-preferring ANA line in several tests of anxiety (Viglinskaya *et al*, 1995; Tuominen *et al*, 1990).

Collectively, the investigation of the effects of ethanol upon behaviour in rodents supports the hypothesis that ethanol elicits an anxiolytic-like effect in behavioural tests of anxiety. However, the dose-dependent effects of ethanol upon locomotor activity in both rats (sedatory) and mice (stimulation) appear to confound the investigation of the anxiolytic properties of the drug in behavioural tests. Therefore, the neuronal mechanisms which underpin the anxiolytic properties of ethanol remain to be determined.

2. Materials and Methods

2.1. Animals

Male Sprague-Dawley rats (Harlan, UK) weighing 250-300 g (unless stated) at the beginning of the experiment were used throughout. Male rats were used in the present study as Nomikos and Spyraki (1988) demonstrated that behaviour in the elevated plus-maze and sensitivity to the anxiolytic effects of diazepam are influenced by the stage of the ovarian cycle in female rats.

Upon arrival, animals were housed in cages of 4 and were habituated to the vivarium for 7 days prior to the start of the experiment. Those that received surgery were housed individually following the procedure to minimise the risk of post-operative infection. The vivarium was maintained at 21 ± 2 ℃ and 55 % humidity with a 12 hr light-dark cycle (lights on at 0600 hr and off at 1800 hr). Water and food were available *ad libitum* in the home cages. Behavioural testing was performed between 1300 hr and 1800 hr. All procedures were performed in accordance with the Animals (Scientific Procedures) Act, 1986 (UK), under the auspices of Project Licences PIL 60/3575 and PIL 60/4289 and the personal license 60/12496.

2.2. Stereotaxic surgery

2.2.1. Background

The word stereotaxis originates from the Greek words *stereos* which means ‘3D’ and *taxis* which means ‘an orderly arrangement’. The term was initially coined by Horsely and Clarke (1908) to describe a novel method of accurately orientating a moveable object into a specific three dimensional location within the brain. Initially pioneered in primates, the stereotaxic instrument of Horsely and Clarke (1908) was later adapted for rodents and allows for the implantation

of guide cannulae, electrodes and microdialysis probes into discreet brain nuclei. In rodents, the stereotaxic instrument or “frame” consists of a base plate, an incisor bar, an anaesthetic nose cone, two ear bars and a manipulator which can be orientated in the anterior-posterior, medial-lateral and dorsal-ventral planes (*figure 2.1.*). The head of the animal is affixed to the frame by two tapered ear bars which fit into the external auditory meati and the incisor bar which rests behind the upper incisors (Cooley and Vanderwolf, 2005). The head is clamped to the incisor bar by sliding the anaesthetic nose cone over the snout.

The stereotaxic surgical technique is based upon the definite and predictable locations of target nuclei relative to the surrounding skull. When affixed to the frame, the skull of the animal remains in a definitive spatial relation to the frame itself. A direct line from one auditory meatus to the other, referred to as the interaural line, was the basic reference point in the Horsely-Clarke method (1908). The three dimensional location, or co-ordinates, of the target nucleus within the brain can be determined relative to the central point of the interaural line (referred to as stereotaxic zero; figure 2.1). The co-ordinates of the target nucleus can be determined from a stereotaxic atlas, such as Paxinos and Watson (1998) or König and Klippel (1963). However, an alternative method was proposed by Hess (1957), who used the anatomical features of the skull as the stereotaxic zero point. Therefore, the modern technique is the amalgamation of both the Hess and Horsely-Clarke methods. Bregma is defined as the intersection of the coronal suture of the skull with the sagittal suture whereas lambda is the intersection of the lambdoidal suture and the sagittal suture (*figure 2.1.;* Cooley and Vanderwolf, 2005). The use of bregma as the reference point is advantageous as the variability of sex, strain and weight is

reported to be minimised by the use bregma as opposed to the interaural line (Whishaw *et al*, 1977; Paxinos *et al*, 1985).

2.2.2. Protocol

Stereotaxic coordinates were verified histologically in cadavers before each set of experimental cannulations. The animals were anaesthetised by inhalation of 5% isoflurane (Baxter International Inc., UK) in medical oxygen and were positioned in a stereotaxic frame (dual lab stereotaxic instrument; Stoelting Ltd.). In addition, the non-steroidal anti-inflammatory carprofen (Rimadyl; Pfizer, UK), a COX-2 inhibitor, was administered subcutaneously to provide analgesia (5 mg/kg; 1 ml/kg). The body temperature of the animal was maintained at 35 ℃ by a thermostatically controlled heat pad (Harvard homeostatic blanket; Harvard Apparatus, UK).

The hair was removed from the scalp and chlorhexidine gluconate solution (Hydrex Pink; Ecolab Ltd., UK) applied to reduce the likelihood of infection. An incision was made along the midline of the scalp to expose the skull and the incisor bar adjusted to ensure that bregma and lambda were level in the dorsal-ventral plane (within ± 0.2 mm).

For cannulation of the DRN, an 8 mm-long stainless steel guide cannula (26 ga; Cooper's Needle Works Ltd., Birmingham, UK; *section 2.3.2*) was mounted on the stereotaxic manipulator at an angle of 26° relative to the vertical plane. Conventionally, the location of the target site is determined from a single reference point (Whishaw *et al*, 1977). However, in the present study the distribution of the cannulae targeted at the DRN was variable in the anterior-posterior plane when initially measured from bregma alone. In agreement, Paxinos *et al*. (1985) suggested that the interaural line may serve as more

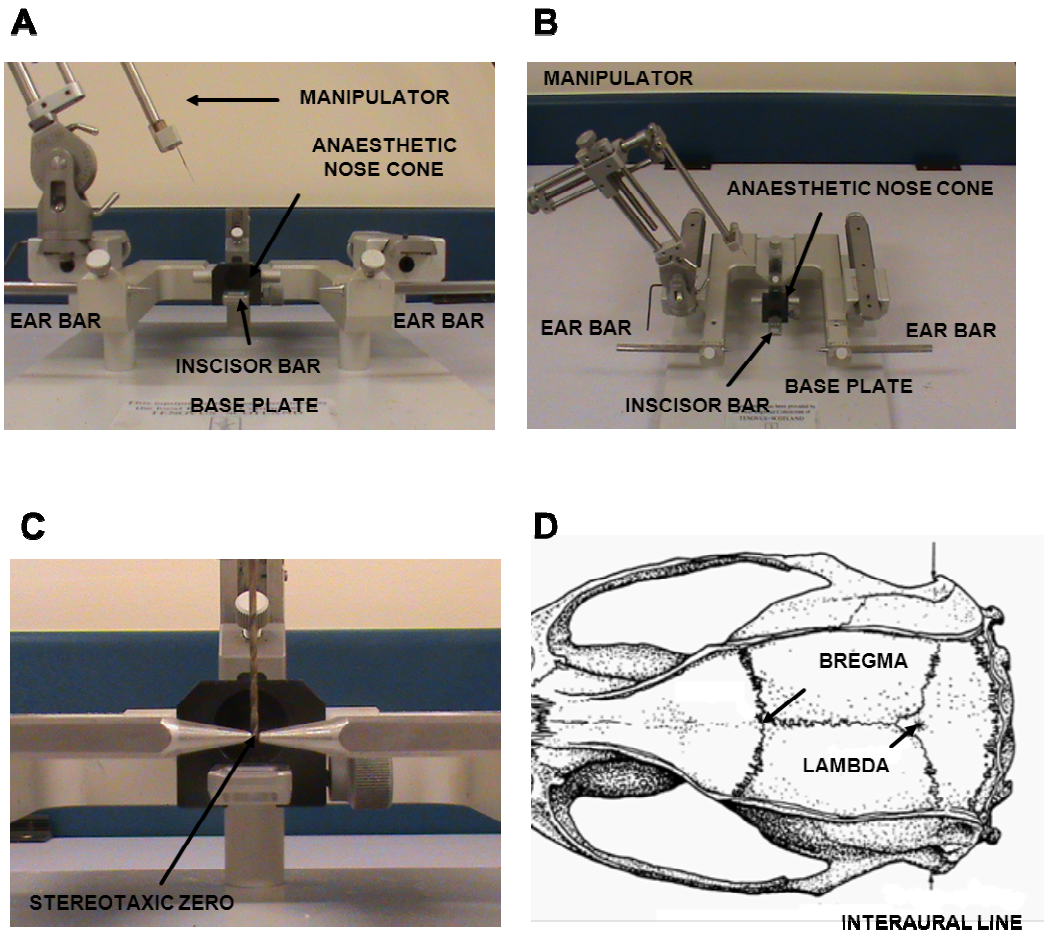

Figure 2.1. – The stereotaxic frame and the reference points for stereotaxic coordinates. The frame itself (**panel A** and **panel B**) consists of a base plate, two ear bars, an incisor bar, the anaesthetic nose cone and a manipulator arm. The head of the animal is held in a fixed position relative to the frame via the ear bars, incisor bar and anaesthetic nose cone. The latter of which provides the anaesthetic required to maintain anaesthesia throughout the procedure. The guide cannula, which is implanted into the brain, is affixed to the manipulator. Therefore, the cannula can be manoeuvred in the anterior-posterior, the lateral and dorsal-ventral planes to the target site. The location of the target site can be determined relative to either stereotaxic zero (**panel C**), bregma or lambda (**panel D**). **Panel D** adapted from Paxinos and Watson (1998).

reliable reference point for caudal structures. Therefore, the anterior-posterior co-ordinate for the DRN was determined as a ratio of the measured distance between bregma and lambda. In accordance with the stereotaxic atlas of Paxinos and Watson (1998), the target site within the DRN is located 7.8 mm posterior to bregma and 1.2 mm anterior to lambda. In the medial-lateral axis, the cannula was positioned 2.9 mm from the midline. The reference point in the

dorsal-ventral axis was taken from the surface of the dura. Three indentations were made in the surface of the skull to accommodate screws (0-80 x 1/8; Bilaney Consultants, UK) implanted in the cranial bones surrounding the target site. An incision in the dura was made using a hypodermic needle and the cannula was lowered 4.1 mm from the surface of the dura.

For cannulation of the lateral ventricle, an 8 mm-long stainless steel guide cannula (26 ga; Cooper's Needle Works Ltd., Birmingham, UK; *section 2.3.2*) was mounted on the stereotaxic manipulator in the vertical plane (i.e. an angle of 0°). The cannula was implanted, as above, and was positioned -0.9 mm posterior from bregma, +1.3 mm lateral to the midline and -2.0 mm below the surface of the dura.

Dental cement (Poly-F Plus Cement; Dentsply, Germany) was built-up to encompass the cannulae and screws in a cement cap and secure the cannulae in position. The patency of the guide cannulae were maintained by the insertion of stainless-steel stylets (34 ga; Cooper's Needle Works Ltd., Birmingham, UK; *section 2.3.2*). Upon termination of the anaesthetic, the animals were returned to the home cage which, in turn, was placed within a heated chamber to aid recovery (27°C; Scanbar A/S, Denmark). Animals were allowed to recover for 10 days prior to the microinjection of substances. The animals were weighed and the stylets replaced daily to accustom the animals to handling and maintain the patency of the cannulae.

2.3. Intracerebral Microinjection

2.3.1. Background

The systemic administration of substances is useful in assessing the effects of a compound on the animal as a whole. However, the substance may not readily cross the blood-brain barrier or may have deleterious consequences when administered systemically. Therefore, investigation of the effects of such compounds in the brain necessitates their administration centrally. In addition, central administration can be used investigate the effects of a substance on discreet nuclei and, within limits, inferences can be made to the effects of the compound on neuronal targets *in vivo* if the pharmacology of the substance has been previously established (e.g. receptor activation, blockade or allosteric modulation).

Historically, Hashimoto (1915; reviewed by Greenshaw, 1986) originally proposed a basic cannula system for the administration of substances into the diencephalon of unanesthetized rabbits. However, the modern technique is based upon the more refined approach of Euler and Holmgren (1956). Guide cannulae are implanted stereotaxically under anaesthesia and are fitted with a stylet to maintain patency. After recovery, the stylet is replaced by a narrower cannula (the microinjection needle) which projects a pre-determined length beyond the tip of the guide cannula into the region of interest. A set volume of solution is slowly administered, via the microinjection needle, into the region of interest using a motorised perfusion pump.

2.3.2. Protocol

Guide cannulae were constructed from 26 ga. stainless steel tubing cut to 8 mm lengths (*figure 2.2*). The guide cannula was tapered at the ventral end, which is stereotaxically implanted into the brain. However, the smooth stainless steel tubing does not provide a reliable adhesive surface for the dental cement.

Therefore, any downward pressure could potentially displace the cannula from its location and damage the target site. To prevent this, shallow grooves were cut into the dorsal half of the cannula to provide an adhesive surface for the dental cement to anchor to (*figure 2.2*).

A stylet was constructed for each guide cannula from 34 ga. stainless steel tubing (*figure 2.2.*). Each stylet was positioned within the guide cannula and protruded 0.1 mm from the ventral tip. The remainder of the stylet, which projects from dorsal tip of guide cannula, is bent to a 45° angle to prevent the stylet protruding further. The lumen of the stainless steel tubing was occluded at both ends. This ensured that, when positioned in the guide cannula, the stylet reduced the necrosis caused by exposure of the cerebral tissue to the open air. The guide cannulae and stylets were autoclaved prior to surgery to minimise the risk of infection.

The main cannula of the microinjection needle was constructed from a 36 ga. stainless steel tube cut to a length of 20 mm (Cooper's Needle Works Ltd., Birmingham, UK; *figure 2.2.*). A stainless steel collar (25 ga.; 3 mm; *figure 2.2.*) was then positioned 10 mm or 9.5 mm from the tip of the main cannula for administration into the DRN and lateral ventricle respectively. This collar serves as a barrier to ensure the needle projects the designed length from the guide cannula tip to the target site. A coil of stainless steel wire (2 mm long coil, 0.2 mm diameter; Advent Research Materials, UK) was positioned above the first collar to act as an adhesive surface to connect all of the components of the microinjection needle (*figure 2.2.*). In addition, a second collar (23 ga.; 3 mm; *figure 2.2.*) with a tapered end was positioned above the coil of stainless steel which was used to connect the needle to the perfusion system. Superglue (Loctite Super Glue Precision; Henkel Loctite) was used to bind the three

external components to the main cannula of the microinjection needle. The depth with which the microinjection needle protrudes beyond the tip of the guide cannula was selected (2 mm for the DRN and 1.5 mm for the lateral ventricle) to minimise the backward diffusion of administered substances into the space between the guide cannula and microinjection needle (Greenshaw, 1986). However, it should be noted that backward diffusion of substances along the outer wall of the microinjection needle is unavoidable as this route offers the least resistance to the solution upon administration into the neuronal tissue.

The delivery of the experimental solution was mediated by a motorised perfusion pump (Pump 22; Harvard Apparatus, UK) which controlled the plunger of the microinjection syringe (2.5 ml; CMA/Microdialysis Type 1, Exmire). The portex tubing (internal diameter 0.58 mm, external diameter 0.98 mm; Smiths Medical International Ltd., UK; *figure 2.2.*), which connected the microinjection syringe and the microinjection needle, was divided into a long and a short section which were connected via a stainless steel junction (23 ga.; 5 mm; *figure 2.2.*). The long section of portex tubing connected the microinjection syringe to the junction and was filled with aCSF solution. The short section of portex tubing was filled with the experimental solution (i.e. vehicle or drug treatment) and was attached to the junction and the microinjection needle. In addition, a small air bubble was incorporated into the short section of portex tubing to separate the experimental solution from the aCSF solution (*figure 2.2.*). The incorporation of the air bubble served to reduce the volume of the experimental solution required and indicated the movement of the experimental solution through the portex tubing.

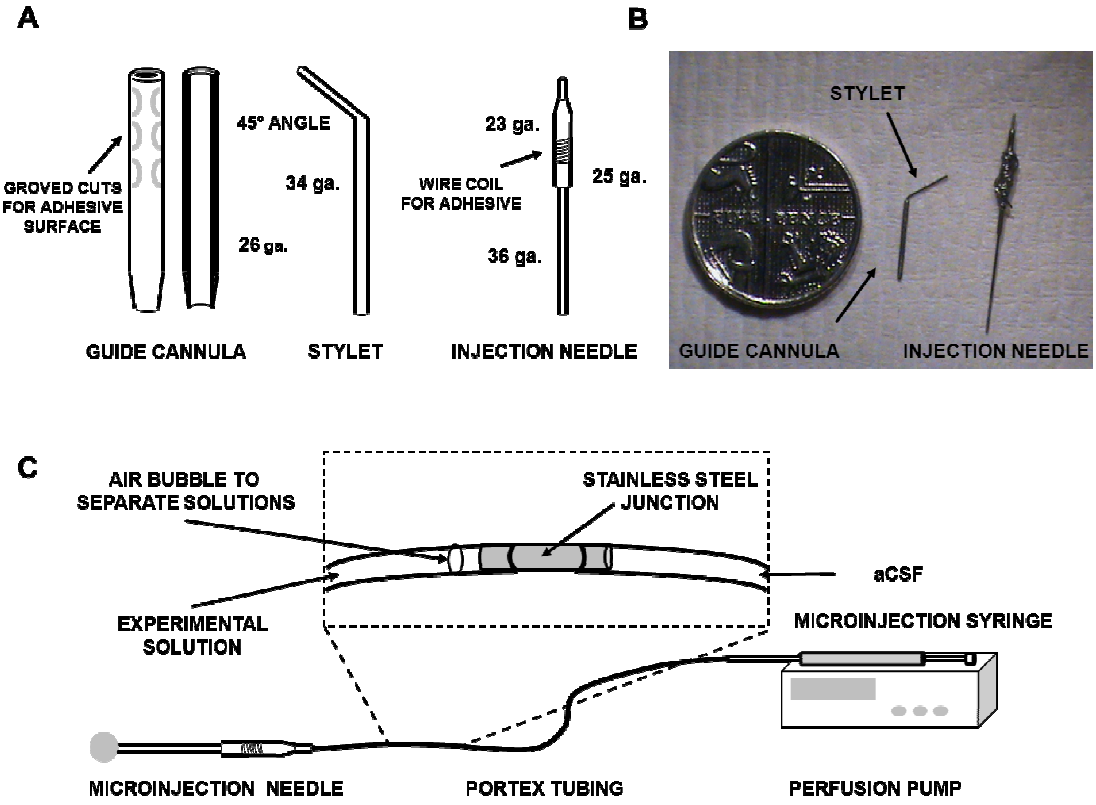

Figure 2.2. – The guide cannula, stylet, microinjection needle and the perfusion system. The guide cannula is constructed from an 8 mm long, 26 ga. stainless steel tube and is surgically implanted into the animal. Post-surgery, a 34 ga. stainless steel stylet is inserted into the guide cannula to maintain patency and prevent tissue necrosis (**panel A** and **panel B**). The administration of the experimental solution is mediated by an aCSF-filled microinjection syringe which is driven by a motorised perfusion pump. The former is connected to the microinjection needle via portex tubing (**panel C**). The portex tubing is divided into a short and long section, which are connected via a stainless steel junction (5 mm, 23 ga.). The shorter section of portex tubing is filled with the experimental solution, while the longer section is filled with aCSF solution. A small air bubble is incorporated into the short section of portex tubing which serves to separate the solutions. On the test day, the stylet is replaced by the 34 ga. microinjection needle which projects beyond the tip of the guide cannula into the target site and the experimental solution is administered.

On the test day, animals were removed from the home cage and gently wrapped in a cloth. The stylet was removed from the guide cannula and replaced with the microinjection needle. The experimental substances were administered at a rate of 250 nl/min to minimise the damage at the target site

caused by displacement. The total volume administered was 1 µl and 500 nl for the lateral ventricle and DRN respectively. The microinjection needle remained in place for a further 2 min following the termination of administration to allow for the diffusion of substances from the needle tip. The microinjection needle was removed and replaced with the stylet. Immediately following the drug administration to the target site, animals were removed from the cloth and tested in the appropriate behavioural apparatus.

2.4. Optimisation of the Intracerebral Microinjection Technique

During the first 12 months of the thesis, considerable effort was made to optimise the surgical, microinjection and histological protocols used in the present study in an attempt to minimise the number of animals used throughout.

Prior to the initiation of experimental studies, 16 cadavers were used to optimise the stereotaxic coordinates. Initial coordinates were based upon Figure 51 of the stereotaxic atlas of Paxinos and Watson (1998). As described in section 2.2.2., the location of the DRN was determined relative to both bregma and lambda. Following the cannulation of a further 10 cadavers it was noted that the use of bregma as a point of reference alone resulted in a high degree of variance in the location of the cannula in the anterior-posterior plane. As the dorsal-ventral position of the DRN varies through the anterior-posterior axis, this variance resulted in a number of misplaced cannulations. Furthermore, the guide cannula was implanted at an angle of 26°. The angle was selected as initial cannulations were implanted at an angle of 10°, however, these were frequently found to occlude the cerebral aqueduct.

The surgical protocol adopted in the present study was predominantly based upon the protocol described by Cooley and Vanderwolf (2005) and remained

consistent throughout. However, alterations were made to improve the recovery of the animals. Following the implantation of the cannulae, the skin was replaced over the dental cement and sutured in place. However, sutures were affixed using either a horizontal or vertical mattress to ensure that the knot of the suture was located away from the incision. This greatly decreased the propensity of the animals removing the sutures.

The microinjection technique was initially based upon the protocol used to administer substances into the DRN reported by File and Gonzalez (1996). However, in a study of 43 rats it was found that the use of a 21 ga. guide cannula and associated 30 ga. microinjection needle resulted in an adverse 'startle-like' reaction which appeared to be due to the mechanical disruption of the target site upon insertion of the microinjection needle into the cannula. Therefore, the microinjection needle and cannula were refined to 34 ga. and 26 ga. respectively and this reduced the incidence of the adverse reaction to the insertion of the microinjection needle.

In addition, the length of the guide cannulae were modified in order to minimise the incidence of the animals removing the stainless steel stylet used to maintain the patency of the guide cannula. Initially, the guide cannulae were 12 mm in length and therefore 8 mm of the cannula protruded from the surface of the skull. However, in the aforementioned study of 43 animals the stainless steel stylet was removed with a regular frequency and led to a number of the cannulae becoming blocked. Attempting to address this issue, commercially manufactured guide cannulae (Bilaney Consultants, UK) were purchased which were fitted with a screw on dust cap. However, an additional 8 animals were able to remove these dust caps with regularity thereby exposing the cerebral tissue at the base of the cannula. This issue was ultimately rectified by reducing

the length of the guide cannula to 8 mm which resulted in 4 mm of the guide protruding from the surface of the skull. This ensured that there was a sufficient surface area for the dental cement to adhere to; however, the guide cannulae protruded a minimal distance from the surface of the cement cap. Furthermore, the construction of the dental cement cap also was integral to the resolution of this problem. As the guide cannula no longer protruded far from the surface of the skull, the cement was built up to encircle the guide cannula rendering the cannula and stylet inaccessible to the paws of the animal while ensuring that the cannula was accessable to the experimentor.

2.5. Drugs

2.5.1. Vehicle solutions

For ICV and intra-DRN administrations, experimental substances were dissolved in an aCSF vehicle (containing, in mM, 126 NaCl, 26 $NaHCO_3$, 10 $MgSO_4$, 10 Glucose, 2.5 KCl, 1.25 NaH_2PO_4 and 0.5 $CaCl_2$; pH 7.4). When bubbled with carbogen (95% oxygen; 5 % carbon dioxide), the aCSF vehicle serves as a buffer to control for changes in pH. The aCSF solution was filtered prior to the addition of the drug using a syringe driven filter (0.2 µm; Millex Syringe Driven Filter Unit). For intraperitoneal administration, experimental substances were dissolved in 0.9 % NaCl solution.

2.5.2. Strychnine

The glycine receptor antagonist strychnine hydrochloride (1 µg/500 nl; Sigma Aldrich, UK) was dissolved in aCSF and administered into the DRN over 2 min. Previously, the effects of intra-DRN administration of strychnine have not been investigated. However, the effects of varying concentrations of centrally

administered strychnine have been investigated in the amygdala (167 pg; McCool and Chappel, 2007), the ventral tegmental area (3 ng; Li *et al*, 2012), the periaqueductal grey (334.4 ng – 668.8 ng; Palazzo *et al*, 2009), the caudal pontine reticular formation (1.67 µg – 3.34 µg; Koch and Friauf, 1995) and the nucleus accumbens (5 µg; Chau *et al*, 2010).

2.5.3. Chlordiazepoxide

In rats, the anxiolytic effects of systemically administered chlordiazepoxide have been investigated extensively in the elevated plus-maze (Criswell *et al*, 1994; Garcia *et al*, 2011; Pellow *et al*, 1985; Moy *et al*, 1997). In the present study, chlordiazepoxide (2.5 mg/kg and 5 mg/kg; chlordiazepoxide hydrochloride; Sigma Aldrich, UK) was dissolved in saline solution and administered intra-peritoneum at 3 ml/kg body weight, 20 min prior to behavioural testing. Therefore, the doses of chlordiazepoxide administered were within the range previously reported to elicit an anxiolytic-like effect in the elevated plus-maze (1.5 – 7.5 mg/kg; Garcia *et al*, 2011; Pellow *et al*, 1985).

2.5.4. Glycine

In the present study, 1 ng, 11 ng and 112 ng of glycine (glycine sodium salt; Sigma-Aldrich, UK) were administered into the DRN (500 nl) over 2 min. Yang *et al*. (1992) reported that the administration of 10 µg glycine into the DRN elicited a decrease in heart rate and mean arterial blood pressure in anaesthetised rats. However, previous studies have administered lower doses of glycine into the hypothalamic paraventricular nucleus (375 ng – 3.75 µg; Krowicki and Kapusta, 2011) and the periaqueductal grey (3 µg - 6 µg, Schmitt *et al*, 1995; 375 ng – 1.5 µg, Palazzo *et al*, 2009). Recently, Li *et al*. (2012) reported a strychnine-sensitive reduction in ethanol consumption in rats

administered low doses of glycine into the ventral tegmental area (37.5 pg – 1.87 ng). Therefore, lower doses were administered in the present study comparable to Li *et al*. (2012) as opposed to the higher dose reported by Yang *et al*. (2002) with consideration of the potential non-specific effects such as increased osmotic pressure.

2.5.5. Ethanol

In the present study, the behavioural effects of ethanol (Ethanol Absolute AnalaR Normapur; VWR International) administered systemically and centrally were investigated. For intra-peritoneum administration, ethanol (0.5 g/kg, 0.75 g/kg, 1.0 g/kg and 1.5 g/kg) was dissolved in saline and administered 6 ml/kg body weight, 20 min prior to behavioural testing. For ICV and intra-DRN administration, ethanol was dissolved in aCSF solution. 128 µg of ethanol (1 µl) was administered into the lateral ventricle over 4 min. In addition, 4 µg, 8 µg and 16 µg of ethanol (500 nl) were administered into the DRN over 2 min.

2.6. Open field and locomotor activity box

2.6.1. Background

The origin of the open field is attributed to Hall (1934) who reported the number of defecations as an index of 'timidity'. The open field is a novel environment consisting of an open area enclosed by walls to prevent escape (*figure 2.3.*). In rats, approach-avoidance conflict is generated by the drive to explore the novel environment and the innate fear of the open central space. Therefore, animals preferentially explore the open field in close proximity to the outer walls as opposed to the centre of the arena (Walsh and Cummings, 1976). Validation of the open field as a measure of anxiety-like behaviours has been reported using

benzodiazepines such as chlordiazepoxide (Sanger and Zivkovic, 1988; Britton and Britton, 1981; Horvath *et al*, 1992) and diazepam (McNamara and Skelton, 1992; Schmitt and Hiemke, 1998; Rex *et al*, 1996). However, the lack of standardisation in the protocols used for the open field is a limitation. For example, the dimensions of the maze itself can vary considerably from wall height, size of arena, shape (circular, square or rectangular), illumination, colour, texture and the starting position of the animal (centre versus corner; Walsh and Cummings, 1976; Stanford, 2007).

2.6.2. Behavioural Measures

Classically, the number of defecations and locomotor activity of the animal were considered measures of emotionality in the open field (Walsh and Cummings, 1976). However, correlation studies have shown that the two measures appear unrelated (Pare *et al*, 1964). Since then numerous measures, including spatiotemporal indices, have been adopted to the protocol. The majority of the 30 or so measures that have been employed are some measure of locomotor activity (Walsh and Cummings, 1976). The most commonly used indices of anxiolytic-like behaviours in the open field are the time spent exploring the central zone, the ratio of central: total locomotion and the latency to enter the central area (Prut and Belzung, 2003). An increase in exploration of the central area in the absence of an overt change in general locomotor activity is interpreted as an anxiolytic-like effect. Locomotor activity, as measured by the number of line crossings, is typically used to investigate putative stimulatory or sedatory effects (Prut and Belzung, 2003). Therefore, in the open field it may be difficult to dissociate between the locomotor and anxiety properties of a substance (Stanford, 2007). In the present study, the percentage of the trial time spent in the central zone and the number of transitions into the central

zone are considered measures of anxiety-like behaviours. Freezing or time immobile is commonly used as an index of anxiety-like behaviour and is thought to reflect a high anxiety state (Walsh and Cummings, 1976). However, this measure can be confounded by periods of sleep which can result from habituation to the environment (Prut and Belzung, 2003).

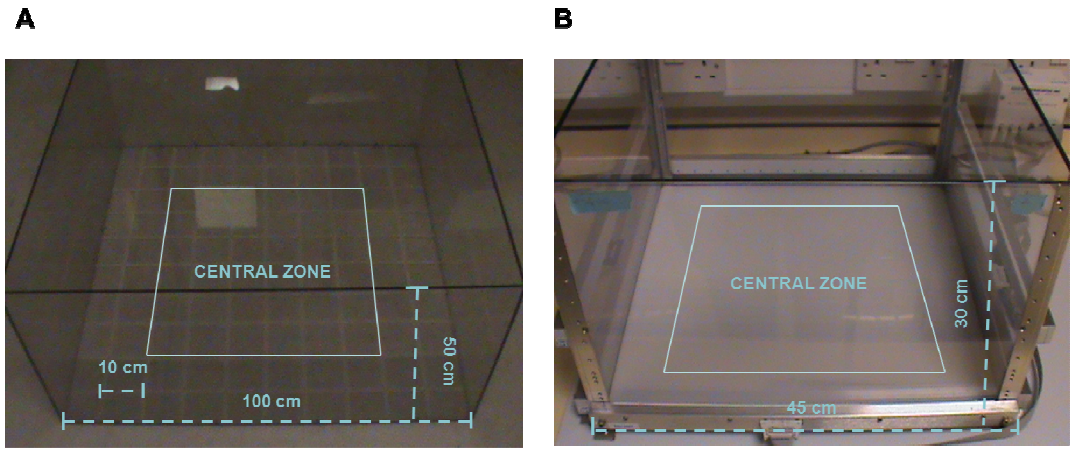

Figure 2.3. – The open field and locomotor activity box. The open field (**panel A**) is a clear perspex box with a 100 cm squared base and 50 cm high walls. The base of the open field was subdivided into 10 cm squares. The total number of line crossings was considered a measure of the general locomotor activity of the animals. The percentage of the trial time spent exploring the innermost 60 cm squared area, termed the central zone, is considered a measure of anxiety-like behaviour. In addition, the number of transitions from the peripheral area into the central zone was used as an index of anxiety. The locomotor activity box (**panel B**) consisted of a 45 cm square base lined with two sets of parallel photobeams (2.54 cm apart) located on adjacent walls. The walls themselves were 30 cm high. The total number of ambulatory counts was determined by the Activity Monitor software and is considered a measure of general activity. In addition, the percentage of the trial time spent exploring the central zone of the arena was considered a measure of anxiety-like behaviour. The central zone was defined as the innermost 11.5 photobeams on either wall (29.21 cm square).

2.6.3. Protocol

On the test day, the rats were moved to the testing room between 0900 hr - 1000 hr to habituate. The open field (*figure 2.3.*) was positioned directly beneath a ceiling-mounted digital camera and the light level in the room was measured at 30 lux. All behavioural testing was performed between 1300 hr - 1800 h.

Following the administration of the experimental solutions, the animals were placed at the centre of the open field as previous studies have shown that animals placed in close proximity to a wall favour the exploration of the area in which they were originally situated (Satinder, 1969). Behaviour was recorded on a digital camera for 15 min and scored as 3 x 5 min time bins. At the end of the trial the animals were removed from the open field apparatus and killed by cervical dislocation.

Similarly, animals were placed in the centre of the locomotor activity box (*figure 2.3.*) for 20 mins. Behaviour in the locomotor activity box was scored by the Activity Monitor software, which tracked the location of the animals via the breaking of photobeams and registered the number of ambulatory counts and the duration of time spent in the central zone. At the completion of the trial, the animals were removed from the locomotor activity box and returned to the home cage.

2.7. Elevated plus-maze

2.7.1. Background

Handley and Mithani (1984) developed the elevated plus-maze based upon the observations of Montgomery (1955), who reported that rats exposed to a three armed maze explored the enclosed arms and avoided the open arms. The elevated plus-maze (*figure 2.4.*) consists of four arms which are elevated from the floor. Two arms, which are situated opposite one another, are walled. The remaining two are open. In the elevated plus-maze conflict is generated by the desire to explore and the innate fear of the elevated open runways (Handley *et al*, 1993). When exposed to the elevated plus-maze animals preferentially explore the enclosed runways as opposed to the open runways. A range of

anxiolytic compounds, such as the benzodiazepines and barbiturates, were found to increase exploration of the open runways, whereas anxiogenic compounds increase avoidance of the aversive environment (Pellow *et al*, 1985). Pellow *et al*. (1985) reported that plasma corticosterone was elevated in animals confined to the open and enclosed runways of the elevated plus-maze when compared to those remaining in the home cage. In addition, the restriction of animals to the open runways was found to increase plasma corticosterone above those confined to the enclosed runways (Pellow *et al*, 1985). Increased expression of Fos protein has been reported in areas of the brain associated with defensive behaviours such as the amygdala, hippocampus, medial hypothalamic area and the periaqueductal grey in response to exposure to the elevated plus-maze (Silveira *et al*, 1993).

2.7.2. Behavioural Measures

Classically, spatiotemporal measures such as the number and percentage of total entries made into the open runways and the percentage of the total time spent exploring the open runways have been reported for the elevated plus-maze. A factor analysis found that these indices load heavily on a single factor interpreted as anxiety and are therefore reported in the present study (Cruz *et al*, 1994). In a similar manner to the open field, measures of anxiety-like behaviour in the elevated plus-maze are dependent on the motor activity of the animals. In agreement, the number of entries into the open runways loads on a second factor in addition to the anxiety factor, which is thought to reflect the motor activity of the animals (Cruz *et al*, 1994). Therefore, the number of open arm entries is predominantly a measure of anxiety but is influenced by the general motor activity of the animals. The number of closed arm entries, as opposed to the total number of arm entries, was reported in the present study

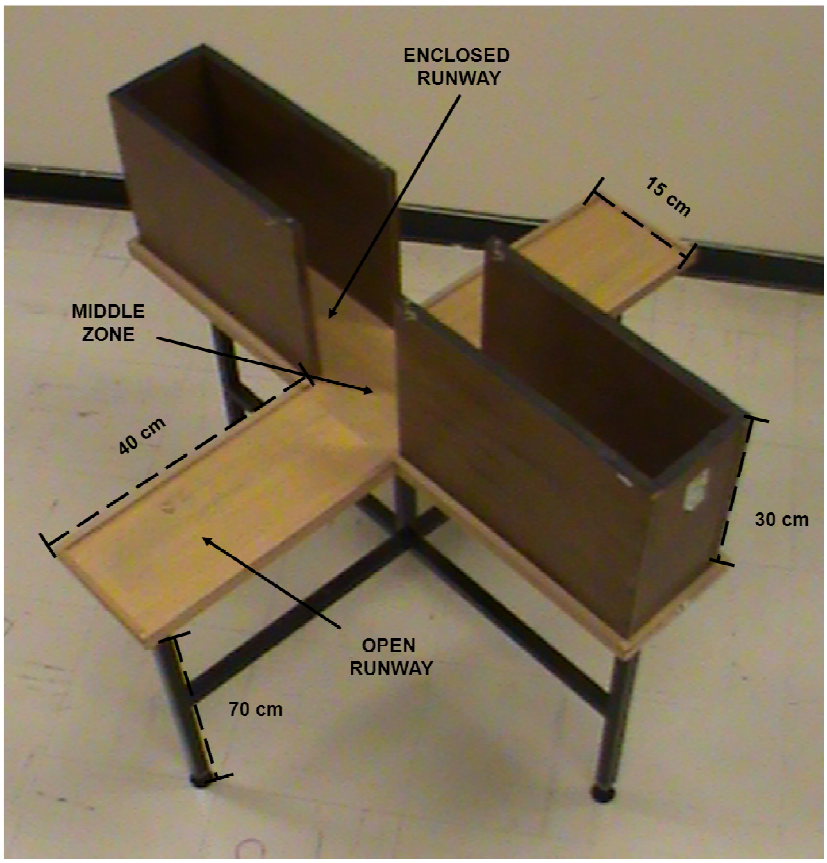

Figure 2.4. – The elevated plus-maze. The plus-maze is a symmetrical maze consisting of four wooden arms elevated 70 cm from the floor. Each arm is 40 cm long and 15 cm wide. Two runways, situated opposite one another, are enclosed with walls 30 cm high. The two remaining arms remain open.

as a measure of locomotor activity. The number of closed arm entries loads heavily on the locomotor activity factor in the factor analysis but does not load on the anxiety factor (Cruz *et al*, 1994). Conversely, the total number of arm entries loads on both the motor and anxiety factors and is therefore a contaminated measure of motility. Additional ethological measures of anxiety-like behaviours, such as risk assessment and avoidance behaviours, in the plus-maze have been proposed (Carobrez and Bertoglio, 2005). However, in rats these measures do not load highly onto the anxiety factor in a factor analysis and were therefore not investigated in the current study (Cruz *et al*, 1994).

2.7.3. Protocol

On the test day, the rats were moved to the testing room between 0900 hr - 1000 hr. The light level at the centre of the elevated plus-maze was measured at 30 lux. The animals were tested in the elevated plus-maze between 1300 hr - 1800 hr. Following the administration of the experimental solution the rat was placed in the centre area of the elevated plus-maze facing an open runway. Pellow *et al*. (1985) have previously reported that rats placed facing an open runway at the beginning of the trial do not show a bias for either the open or enclosed runways when making the initial arm entry. The behaviour was recorded for 15 minutes using a digital camera and was scored as 3 x 5 min time bins (unless stated). The entry of an animal into an arm of the elevated plus-maze was scored if all four paws were moved into the confines of the arm. Following behavioural testing, the animal was removed from the maze and killed by cervical dislocation (unless specified).

2.8. Elevated open platforms

2.8.1. Background

Previously, the open field and elevated plus-maze have been discussed as behavioural tests which investigate behaviour in response to acute stress. However, there are instances where an aversive stimulus cannot be escaped from or avoided and therefore the animal must adapt physiologically to cope with the aversive stimulus. Previous studies suggest that the acute and repeated exposure to an inescapable stressor results in regionally-selective physiological adaptations in the brain which are thought to mediate the process of habituation (Amat *et al*, 2005; Christianson *et al*, 2009; Graeff *et al*, 2004). The process of habituation has been investigated previously in the laboratory

using repeated exposure to an inescapable elevated open platform stressor (Storey *et al*, 2006; Robertson *et al*, 2005; *figure 2.5.*). Acute exposure (1 hr) to the elevated open platform increases plasma corticosterone levels (Storey *et al*, 2006). However, the plasma corticosterone of animals repeatedly exposed to the platforms over the course of 20 days (1 hr daily) was not significantly different from unstressed controls suggesting the habituation of the endocrine response to the stressor (Storey *et al*, 2006).

The processes of habituation to the elevated open platform stressor may be mediated by regionally-selective alterations in serotonergic transmission. Acute exposure to an inescapable stressor evokes an increase in serotonin overflow and tissue levels of the serotonin metabolite 5-HIAA in the frontal cortex (Bland *et al*, 2003; Storey *et al*, 2006). However, the repeated exposure to the elevated open platform (1 hr daily, 10 days) abolishes this increase in serotonin overflow and 5-HIAA levels in the frontal cortex (Storey *et al*, 2006). Therefore, acute exposure to the inescapable stressor elicits an increase in serotonin turnover and release of 5-HT in the frontal cortex. Azmitia and Segal (1978) reported that the serotonergic innervation of the prefrontal cortex originates from the DRN as opposed to the MRN. Therefore, acute exposure to the elevated open platform stressor may increase the neuronal excitation of the serotonergic neurons of the DRN. Previous studies have reported an increase in serotonin overflow in the vmPFC of rats acutely exposed to inescapable shocks (Bland *et al*, 2003; Amat *et al*, 2005). However, the repeated exposure to the inescapable stressor may suppress the firing of the serotonergic neurons originating from the DRN in response to the stressor.

Serotonin overflow and tissue 5-HIAA levels in the dorsal hippocampus were increased in animals repeatedly (10 days, 1 hr daily), but not acutely (1 hr),

exposed to the elevated open platform (Storey *et al*, 2006). In addition, Robertson *et al*. (2005) reported an increase in the expression of mineralocorticoid (MR) and glucocorticoid (GR) receptors in the hippocampus in response to the repeated exposure to the stressor. However, this upregulation of MR and GR was abolished by the selective lesioning of the projections from the MRN to the dorsal hippocampus. Collectively, the results support the dual role of serotonin proposed by Graeff *et al.* (1996) who suggested that serotonergic projections from the MRN to the hippocampus facilitate resistance to chronic stress by disconnecting aversive events from the neurological process which underpin appetitive and social behaviours.

In addition to physiological adaptations, repeated exposure to the elevated open platform stressor results in an anxiolytic-like effect when animals are subsequently challenged in the elevated plus-maze 24 hrs after the final exposure to the platforms. Repeated exposure (10 days) to the elevated open platform stressor significantly increased the number of open arm entries, the percentage of total entries made into the open runways and the time spent exploring the open runways (Storey *et al*, 2006). Previous studies have shown that plasma corticosterone is increased in response to acute exposure to the elevated plus-maze (Pellow *et al*, 1985). However, in animals repeatedly pre-exposed to the elevated open platform stressor for 10 days this increase in plasma corticosterone in response to the elevated plus-maze was enhanced above the levels of animals acutely exposed to the elevated plus-maze alone (Storey *et al*, 2006).

2.8.2. Protocol

Animals in the home cage were transported through to the testing room between 0900 hr – 1000 hr. The lighting in the room was approximately 30 lux (as measured from the centre of the platform). Between 0900 hr and 1200 hr, animals were removed from the home cage and placed in the centre of the elevated open platform for 1 hr. The animals were removed from the platform and returned to the home cage. Animals were pre-exposed to the elevated open platform for 1 hr daily for 10 consecutive days. On the 11th day, animals were tested on the elevated plus-maze as described above.

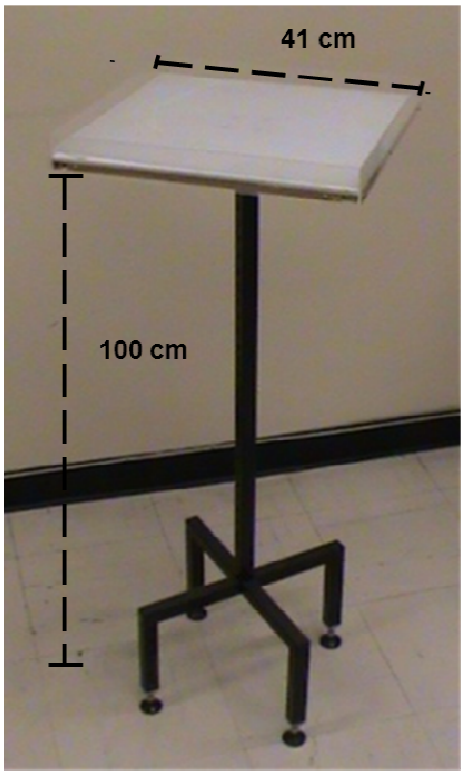

Figure 2.5. – The elevated open platform. The elevated platform consisted of a square podium (41 cm squared) with surrounding walls (2.5 cm high) to minimise the incidence of animals falling from the platform. The podium was attached to the central column, which raised the podium to a height of 1 m. The podium itself was covered with a square sheet of diamond textured perspex to increase grip for the animals.

2.9. Histological identification of the microinjection site

Animals were removed from the behavioural apparatus upon completion of the trial and were killed humanely by cervical dislocation. 2% Chicago sky blue dye (500 nl over 2 min; Sigma-Aldrich, UK; Chau *et al*, 2010), dissolved in the aCSF vehicle, was administered into the DRN via the indwelling cannula to visualise the site of the injection for histological analysis. The brain was dissected from the skull and stored in a 0.2 M phosphate buffered 4% formalin solution for two days. The brain was then transferred to a 0.2 M phosphate buffered 30% surcrose solution for three days prior to sectioning of the tissue.

However, animals which were administered substances into the lateral ventricle were killed humanely by transcardial perfusion. The administration of Chicago sky blue dye was insufficient to identify the location of microinjection sites targeted at the lateral ventricle. Animals were administered Euthital (sodium pentobarbitone; Pfizer, UK) at 2 ml/kg body weight. Upon sedation, the animals received a microinjection of 2% Chicago sky blue dye (1 µl and 500 nl over 2 min for ICV and DRN respectively) via the indwelling cannula. Upon completion of the administration, the microinjection needle was replaced with an elongated stylet. These stylets extended 1.5 mm and 2 mm beyond the tip of the guide cannula to mimic the length of the injector during fixation. The onset of the terminal anaesthesia was confirmed by the absence of the appendage withdrawal reflex in response to a pinch of the tip of the tail and the skin between adjacent toes. The heart was subsequently exposed and a needle was inserted into the left ventricle and clamped using haemostats. Following an incision into the right atrium, ice cold heparinised saline (3 ml heparin/ 100 ml saline; Leo Laboratories, UK) was perfused into the cadaver via the needle. The saline solution was replaced with 0.2 M phosphate buffered 4% formalin solution (12 ml/min). Upon fixation of the cadaver, the brain was dissected from

the skull and stored in 0.2 M phosphate buffered 4% fixative solution for a period of 3 days and was replaced by the 0.2 M phosphate buffered 30% sucrose solution as described previously.

The brains were removed from the 0.2 M phosphate buffered 30% sucrose solution and frozen at -50°C. The brains were cut into 32 µm sections at -20°C using a cryostat (Clinicut 60 Cryostat, Bright Instruments Co Ltd., UK) and mounted on gelatinised microscope slides. To visualise the topography of the brain, the sections were stained with the Nissl stain Cresyl Violet and viewed under the microscope (Axioskop 2; Carl Zeiss Ltd., UK; *figure 2.6.*). Animals with injection sites located out with the target site, as defined by Paxinos and Watson (1998), were removed from the analysis as determined by an

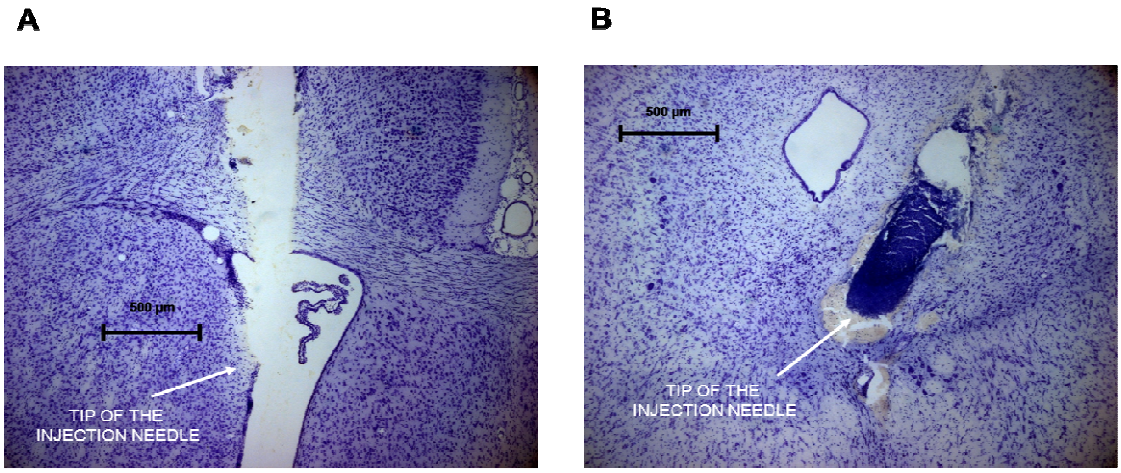

Figure 2.6. – Examples of injection sites located within the lateral ventricle and DRN. Upon completion of behavioural testing, animals were killed humanely by cervical dislocation or transcardial perfusion. Microinjection sites were visualised by the administration of Chicago sky blue dye via the indwelling cannula. The tissue was dissected from the skull and fixed in a 4% formalin solution. The brain was cut to 32 µm coronal sections and mounted on gelatinised microscope slides. To visualise the topography of the brain, sections were stained using cresyl violet and were examined under the microscope. Microinjection sites located within the lateral ventricle (**panel A**) and the DRN (**panel B**) were included in the statistical analysis. However, animals were excluded if the microinjection was located out with the target site. Confirmation of the injection site was verified by an experimenter blind to the treatment and behavioural history of the animals.

experimenter blind to the treatment administered and the behavioural history of the animal.

2.10. Statistical Analysis

Behavioural measures were analysed using repeated measures analysis of variance with time as the within-subjects factor and the treatment(s) as the between subjects factor. Greenhouse-Geisser correction factor applied to data which did not adhere to Mauchly's test of sphericity. Post hoc analysis of the between-subject factors was performed by Games-Howell test. Post hoc analysis of interactions of the between and within-subjects factors were performed using independent samples t-tests.

3. Do the strychnine-sensitive glycine receptors expressed by the serotonergic neurons of the DRN influence anxiety-like behaviours?

3.1. The effect of intra-DRN administration of strychnine on the exploration of the open field.

3.1.1. Rationale

Collectively, the administration of substances which suppress the neuronal excitation of the serotonergic neurons in the DRN are reported to induce anxiolytic-like effects in both conditioned and unconditioned behavioural tests of anxiety (*section 1.4.4.*). In the present study, the selective glycine receptor antagonist strychnine was administered into the DRN to suppress, as opposed to facilitate, the neuronal inhibition of the serotonergic neurons. Prior studies in the laboratory have reported that the neuronal excitation of the serotonergic neurons of the DRN is suppressed by a tonically active strychnine-sensitive glycinergic inhibition (Maguire *et al*, 2013; *section 1.4.5.*). Therefore, the antagonism of the glycinergic inhibition was hypothesised to increase anxiety-like behaviours and this was investigated in the open field.

3.1.2. Methods

Male Sprague-Dawley rats weighing 250-300 g at the beginning of the experiment were implanted with guide cannulae targeted at the DRN (*see section 2.2 and 2.3*). The animals were left for 10 days in the home cage to recover. On day 11 post-surgery, animals were transported to the testing room. Under gentle retraint, the stylet was replaced with a 36 ga. needle protruding 2 mm beyond the tip of the cannula into the DRN. The rats were administered 1 µg strychnine hydrochloride (n=7) or the aCSF vehicle (500 nl; n=9) over 2 min by means of the needle inserted through the guide cannula. The needle was left in place for a further 2 min to allow for diffusion of the solution from the tip of the needle. The animals were then immediately placed at the centre of a perspex

open field (*see section 2.6*). Activity in the open field was recorded on a digital camera for 15 min and scored as 3 x 5 min time bins (number of line crossings, time in the central zone and transitions into the central zone; *figure 3.1*.). Upon completion of the behavioural study the rats were killed humanly by cervical dislocation and the injection site was verified histologically in fixed sections of the DRN by an observer blind to the treatment the rats had received (*figure 3.1*.). The experiment was performed with a single group of 20 animals. Animals were tested over 5 days, with 4 animals tested in the elevated plus-maze on each day. Of the 20 animals which began the experiment, 1 animal could not be administered the experimental solution due to the blockade of the guide cannula. In addition, 3 animals were removed from the study as the histological investigation identified the administrations were located outwith the DRN. The behaviour was analysed by a repeated measures ANOVA with treatment on the test day (strychnine or aCSF) as the between-subjects factor and time as the within-subjects factor (0-5 min, 5-10 min and 10-15 min time bins). Greenhouse-Geisser correction factor applied to data which did not adhere to Mauchly's test of sphericity.

3.1.3. Results

The blockade of the strychnine-sensitive glycine receptors, achieved via the intra-DRN administration of the antagonist strychnine into the DRN, had no significant effect upon the total number of line crossings suggesting the antagonism of the glycine receptors did not elicit an overt change in locomotor activity. Contrary to the hypothesised anxiogenic-like effect, neither the percentage of the total time spent exploring the central zone nor the number of transitions into the central zone were significantly influenced by the intra-DRN administration of strychnine. Therefore, this suggests the antagonism of the

glycine receptors of the DRN did not significantly influence anxiety-like behaviours in the open field. However, both the number of line crossings and the percentage of the trial time spent in the central zone varied significantly between the time bins suggesting the behaviour of the animals in the open field changed over the course of the trial time.

Repeated measures ANOVA showed that strychnine had no significant effects on the total number of line crossings ($F(1,14) = 0.48$; n.s.; *figure 3.2.*), the percentage of the total time spent exploring the central zone ($F(1,14)=2.337$; n.s.; *figure 3.2.*) or the number of transitions into the central zone ($F(1,14)=0.708$; n.s.; *figure 3.3.*).

The total number of line crossings (*Greenhouse-Geisser correction applied*; $F(1.207, 16.894)=26.968$; $p<0.001$) and the percentage of the total time spent exploring the central zone ($F(2,28)=44.121$; $p<0.001$) were significantly different over the course of the time bins. However, the number of transitions from the periphery to the central zone (*Greenhouse-Geisser correction applied*; $F(1.259, 17.625)=2.613$; n.s.) did not significantly differ across the time bins.

There was no significant interaction between the administration of strychnine and the time bins on the total number of line crossings (*time x treatment*; *Greenhouse-Geisser correction applied*; $F(1.207, 16.894)=1.235$; n.s.; *figure 3.2.*), the percentage of the total time spent in the central zone (*time x treatment*; $F(2,28)=0.462$; n.s.; *figure 3.2.*) or on the number of transitions from the periphery into the central zone. (*time x treatment*; *Greenhouse-Geisser correction applied*; $F(1.259, 17.625)=0.680$; n.s.; *figure 3.3.*).

3.1.4. Discussion

Previous studies have reported that the administration of 8-OH DPAT into the DRN mediates an anxiolytic-like effect in the social interaction test (Hogg *et al*, 1994; Higgins *et al*, 1988) and elevated plus-maze (File and Gonzales, 1996). Therefore, it was hypothesised that the administration of strychnine into the DRN would facilitate the neuronal excitation of the serotonergic neurons, via the blockade of the strychnine-sensitive glycine receptors, and this would elicit an anxiogenic-like effect in animals exposed to the open field. However, Higgins and Elliott (1991) reported the intra-DRN administration of the 5-HT_{1A} receptor ligands 8-OH DPAT and gepirone produced a hypoactive state in the open field. The findings of Higgins and Elliott (1991) would suggest that the suppression of the serotonergic neurons of the DRN mediates an anxiogenic-like effect and therefore the administration of strychnine would elicit an anxiolytic-like effect in the open field. The administration of 8-OH DPAT reduced the number of line crossings, the number of rearings and the percentage of the trial time spent in the central zone (Higgins and Elliott, 1991). In addition, there was an increased incidence of a flattened body posture (Higgins and Elliott, 1991). However, the doses of 8-OH DPAT (500 ng and 2.5 µg) administered by Higgins and Elliott (1991) were higher than had previously been reported to elicit anxiolytic-like effects in the social interaction test (20 - 100 ng; Hogg *et al*, 1994; Higgins *et al*, 1988) and elevated plus-maze (100 - 200 ng in maze experienced animals; File and Gonzales, 1996).Therefore, the effects reported by Higgins and Elliott (1991) may be mediated by a non-specific mechanism.

Contrary to the original hypothesis, blockade of the strychnine-sensitive glycine receptors of the DRN via the administration of the antagonist strychnine did not induce an anxiogenic-like phenotype in the open field but had no significant influence on either anxiety-like behaviours or locomotor activity. In the present

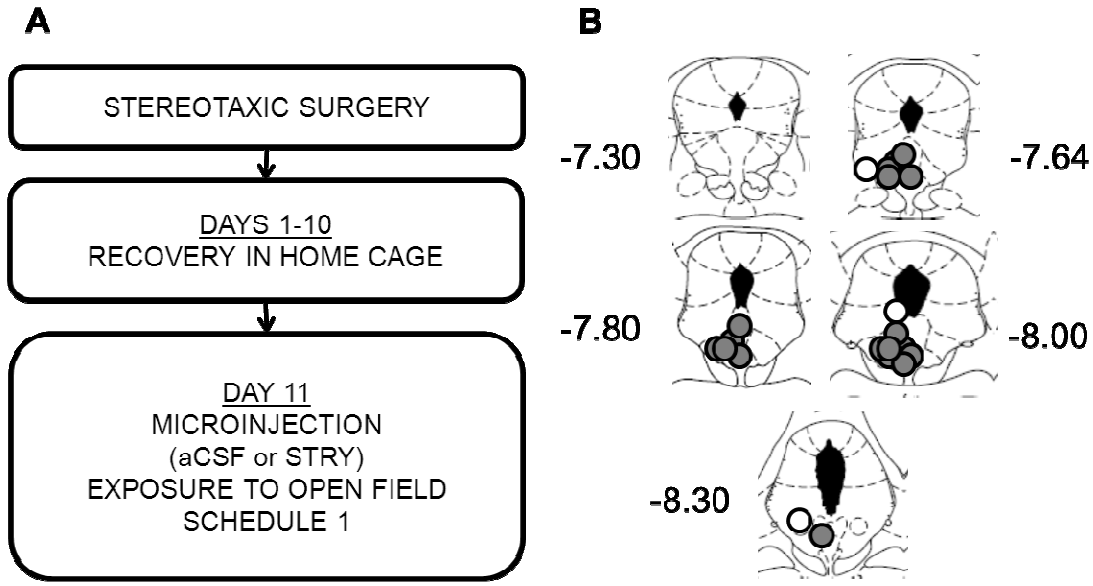

Figure 3.1. – Experimental design and a representative schematic showing the distribution of microinjection sites targeted at the DRN. Panel A shows the experimental design, where the number of days denotes the number of days post-surgery. **Panel B** is a diagramatic representation of the DRN and surrounding area adapted from the coronal sections of Paxinos and Watson (1998). Numerical values indicate the posterior distance from Bregma. Following behavioural testing and cervical dislocation, 2% Chicago sky blue dye was administered into the DRN. The brain was removed and fixed in a 4% formalin solution for 48 hr and then transferred to a 30% sucrose solution for 72 hr. Coronal sections (36 µm) containing the DRN were mounted on gelatinised slides and stained using cresyl violet to visualise the topography of the brain. Filled circles show examples of cannulations which resulted in the successful administration of substances into the DRN and were included in the study. Open circles depict the location of injection sites excluded from the study either due to the administration of substances outside of the DRN or by the occlusion of the cerebral aqueduct. Histological assessment of the cannulations was performed by an observer blind to the treatment administered and behavioural history of the animal.

study, the lack of measurable behavioural outcomes in response to the intra-DRN administration of strychnine may be due to the nature of the treatment. Strychnine is a competitive antagonist of the strychnine-sensitive glycine receptor and occupies a site overlapping the agonist binding site (O'Connor *et al*, 1996; Grudzinska *et al*, 2005). Therefore, behavioural outcomes are dependent upon the extracellular concentration of the endogenous agonist

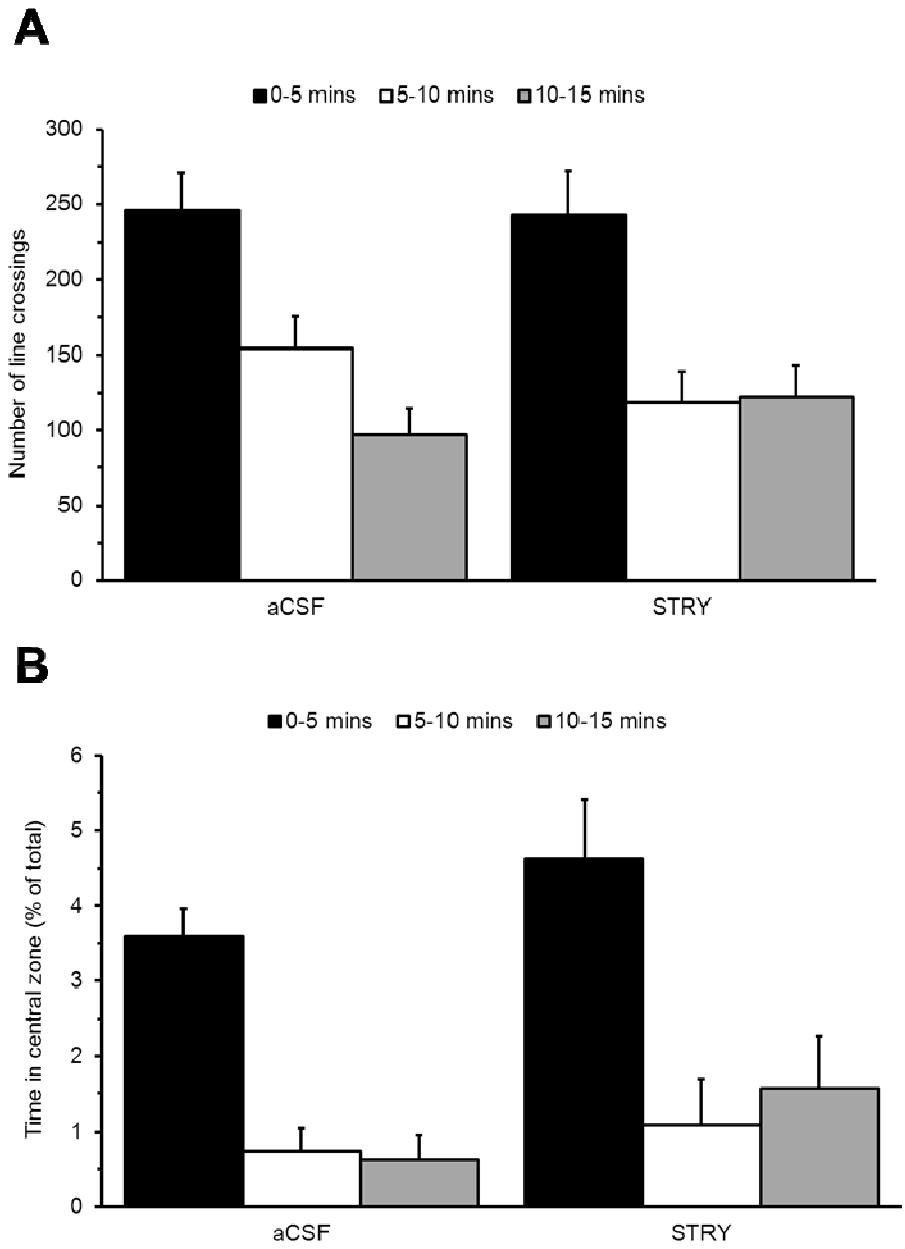

Figure 3.2. – The effect of intra-DRN administration of strychnine on the total number of line crossings and the percentage of the total time spent in the central zone of the open field. On day 11 post-surgery, animals were administered 1 µg strychnine hydrochloride (*n=7*) or the aCSF vehicle (500 nl; *n=9*) into the DRN via the indwelling cannulae prior to a 15 min exposure to the open field. The number of line crossings was not significantly influenced by the administration of strychnine (**panel A**). The percentage of the total time spent exploring the central zone of the open field was not significantly influenced by the administration of strychnine into the DRN (**panel B**). Data are presented as the mean ± S.E.M.

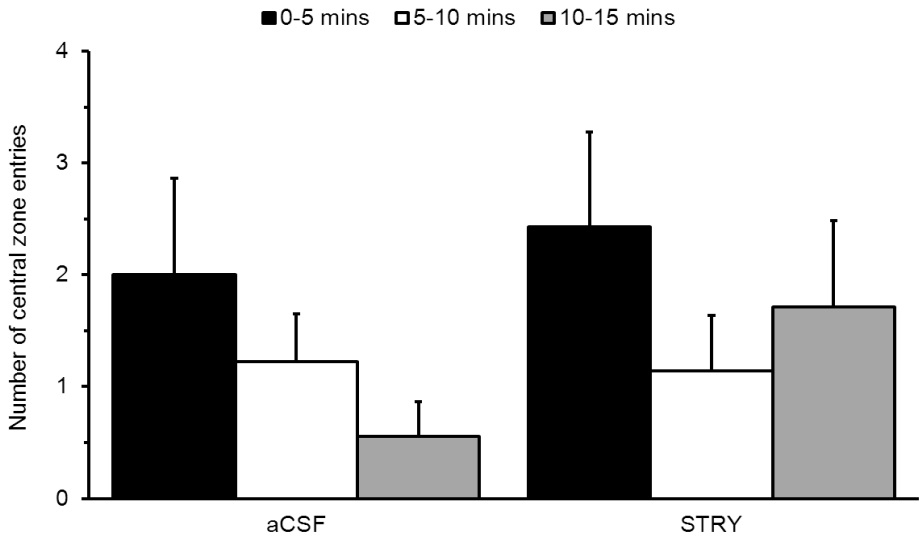

Figure 3.3. – The effect of intra-DRN administration of strychnine on the number of transitions from the periphery into the central zone of the open field. On day 11 post-surgery, animals were administered 1 µg strychnine hydrochloride (*n=7*) or the aCSF vehicle (500 nl; *n=9*) into the DRN via the indwelling cannulae immediately prior to a 15 min exposure to the open field. The number entries made into the central zone of the open field was not significantly influenced by the administration of strychnine into the DRN. Data are presented as the mean ± S.E.M.

within the DRN during the testing period. The low percentage of the total time spent and number of transitions into the central zone in both aCSF and strychnine treated animals would suggest a high basal state of anxiety upon exposure to the open field. As previously discussed (*section 1.3.1*), the behavioural expression of a high anxiety state is associated with increased serotonergic transmission. Therefore, it may be that under such conditions the concentration of the endogenous agonist which acts upon the strychnine-sensitive glycine receptors may be low and this may contribute to the lack of the hypothesised anxiogenic-like effects of the intra-DRN administration of strychnine.

Therefore, the behavioural effects of strychnine may be masked by the high basal levels of anxiety in the animals. A number of factors associated with the experimental protocol may have influenced the basal anxiety state of the animals (*section 5*). However, Hale *et al*. (2008) reported a mild, but statistically significant, regionally-selective increase in c-Fos expression in the serotonergic neurons of the DRN in animals exposed to the open field. Therefore, it is unlikely that exposure to the open field is the sole mediator of the high basal state of anxiety observed.

3.2. The effect of systemic administration of chlordiazepoxide on the exploration of the open and closed arms of the elevated plus-maze.

3.2.1. Rationale

To investigate the putative effects of strychnine-sensitive glycine receptors in the DRN, the elevated plus-maze test of anxiety was used in conjuncture with the open field. The benzodiazepine chlordiazepoxide has been used as part of a battery of anxiolytic compounds, with a known therapeutic value in humans, to demonstrate the validity of the elevated plus-maze as a test of anxiety-like behaviour in rats (Pellow *et al*, 1985). In order to validate the elevated plus-maze methodology used in the present study, the effects of intra-peritoneal (i.p.) administration of chlordiazepoxide on the exploration of the elevated plus-maze was investigated prior to assessing the putative role of the strychnine-sensitive glycine receptors in the DRN.

3.2.2. Methods

Male Sprague-Dawley rats (250-300 g) were housed in cages of four throughout. Animals received daily i.p. injections of saline (1 ml/kg) for 7

consecutive days in the testing room to habituate to the injection procedure. On day 8, animals were transported to the testing room and received an i.p. injection of either 2.5 mg/kg (n=7) or 5 mg/kg chlordiazepoxide (n=8) or the saline vehicle (3 ml/kg; n=10) 20 minutes prior to exposure to the elevated plus-maze. Animals were placed in the centre of the elevated plus-maze facing an open runway (*see section 2.7*). Behaviour in the elevated plus-maze recorded on a digital camera for 15 mins and scored as 3 x 5 min time bins (number of open arm entries, percentage of total entries made into the open runways, percentage of total time spent in the open runways and the number of entries into the closed arms; *figures 3.4. and 3.5.*). The experiment was performed using an original batch of 12 animals and a subsequent batch of 16 animals. Animals were tested over 7 days, with 4 animals tested in the elevated plus-maze on each day. Of the 28 animals which began the experiment, 3 animals were removed from the final analysis after identification as statistical outliers (defined as greater than double the standard deviation from the mean). Data were analysed by repeated measures ANOVA (dose of chlordiazepoxide as the between-subjects factor and time as the within-subjects factor). *Post-hoc* analysis of the between-subject factor was performed using the Games-Howell test. Independent samples t-tests are reported for the investigation of the interactions between the within and between subject factors.

3.2.3. Results

The effect of systemically administering the $GABA_A$ receptor allosteric modulator chlordiazepoxide upon the the number and percentage of entries made into the open runways and the percentage of the trial time spent in the open runways of the elevated plus-maze varied significantly over the course of the time bins. When compared against the saline control, both the 2.5 mg/kg

and 5 mg/kg doses of chlordiazepoxide significantly increased all three indices of anxiety-like behaviour in the initial 0-5 min time bin characteristic of an anxiolytic-like effect. However, the 5 mg/kg dose of chlordiazepoxide significantly decreased the indices of anxiety in the final 10-15 min time bin. In addition, the 5 mg/kg dose of chlordiazepoxide significantly decreased the number of enclosed arm entries during the 15 min trial which suggest a suppression of locomotor activity.

Repeated measures ANOVA showed that chlordiazepoxide significantly influenced the number of open arm entries ($F(2,22)=16.549$; $p<0.001$; *figure 3.4.*), the percentage of total entries made into the open runways ($F(2,22) = 7.053$; $p<0.01$; *figure 3.4.*), the percentage of the total time spent exploring the open arms ($F(2,22) = 9.769$; $p<0.01$; *figure 3.5.*) and the number of closed arm entries ($F(2,22) = 11.964$; $p<0.001$; *figure 3.5.*). *Post hoc* analysis of the main effects of the treatment showed that 2.5 mg/kg ($p<0.01$), but not 5 mg/kg (*n.s.*), chlordiazepoxide significantly increased the number of entries into the open runways (*figure 3.4.*), the percentage of total entries made into the open arms ($p<0.05$; *figure 3.4.*) and the percentage of the total time spent exploring the open runways of the maze ($p<0.05$; *figure 3.5.*) during the 15 min trial. Conversely, the 5 mg/kg ($p<0.01$), but not the 2.5 mg/kg (n.s.), dose of chlordiazepoxide significantly reduced the number of entries made into the enclosed runways of the elevated plus-maze (*figure 3.5.*).

The number of open arm entries ($F(2,44)=40.344$; $p<0.001$), the percentage of the total arm entries made into the open runways ($F(2,44) = 11.564$; $p<0.001$), the percentage of the total time spent in exploring the open arms ($F(2,44) = 18.550$; $p<0.001$) and the number of enclosed arm entries ($F(2,44) = 61.709$; $p<0.001$) varied significantly over the time bins of the trial.

The effect of chlordiazepoxide varied over the time bins and influenced the number of open arm entries (*time x treatment*; $F(4,44)=5.717$; $p<0.01$; *figure 3.4.*), the percentage of the total entries made into the open runways (*time x treatment*; $F(4,44) = 7.192$; $p<0.001$; *figure 3.4.*) and the percentage of the total time spent exploring the open runways (*time x treatment*; $F(4,44) = 5.556$; $p<0.01$; *figure 3.5.*). However, the effect of chlordiazepoxide on the enclosed arm entries did not vary over the course of the time bins (*time x treatment*; $F(4,44) = 0.413$; n.s.; *figure 3.5.*).

Post hoc analysis showed that in the 0-5 min time bin, the administration of 2.5 mg/kg chlordiazepoxide significantly increased the number of open arm entries ($t\,(15) = -5.312$, $p<0.001$; *figure 3.4.*), the percentage of the total arm entries made into the open runways ($t\,(15) = -3.130$, $p<0.01$; *figure 3.4.*) and the percentage of the total time spent in the open runways ($t\,(15) = -3.211$, $p<0.01$; *figure 3.5.*). In the 5-10 min time bin, 2.5 mg/kg chlordiazepoxide significantly increased the number of open arm entries ($t\,(15) = -2.246$, $p<0.05$; *figure 3.4.*) and the percentage of time spent exploring the open runways ($t\,(15) = -2.478$, $p<0.05$; *figure 3.5.*), but had no significant effect on the percentage of the total entries made into the open runways ($t\,(15) = -1.845$, n.s.; *figure 3.4.*). In the 10-15 min time bin, the administration of 2.5 mg/kg chlordiazepoxide had no significant effects on the number of open arm entries ($t\,(15) = -0.466$, n.s.; *figure 3.4.*), the percentage of the total arm entries made into the open runways ($t\,(15) = -0.361$, n.s.; *figure 3.4.*) or the percentage of the total time spent in the open arms ($t\,(15) = -0.299$, n.s.; *figure 3.5.*).

The administration of 5 mg/kg significantly increased the number of open arm entries ($t\,(16) = -2.322$, $p<0.05$; *figure 3.4.*), the percentage of the total entries made into the open runways ($t\,(16) = -3.701$, $p<0.01$; *figure 3.4.*) and the

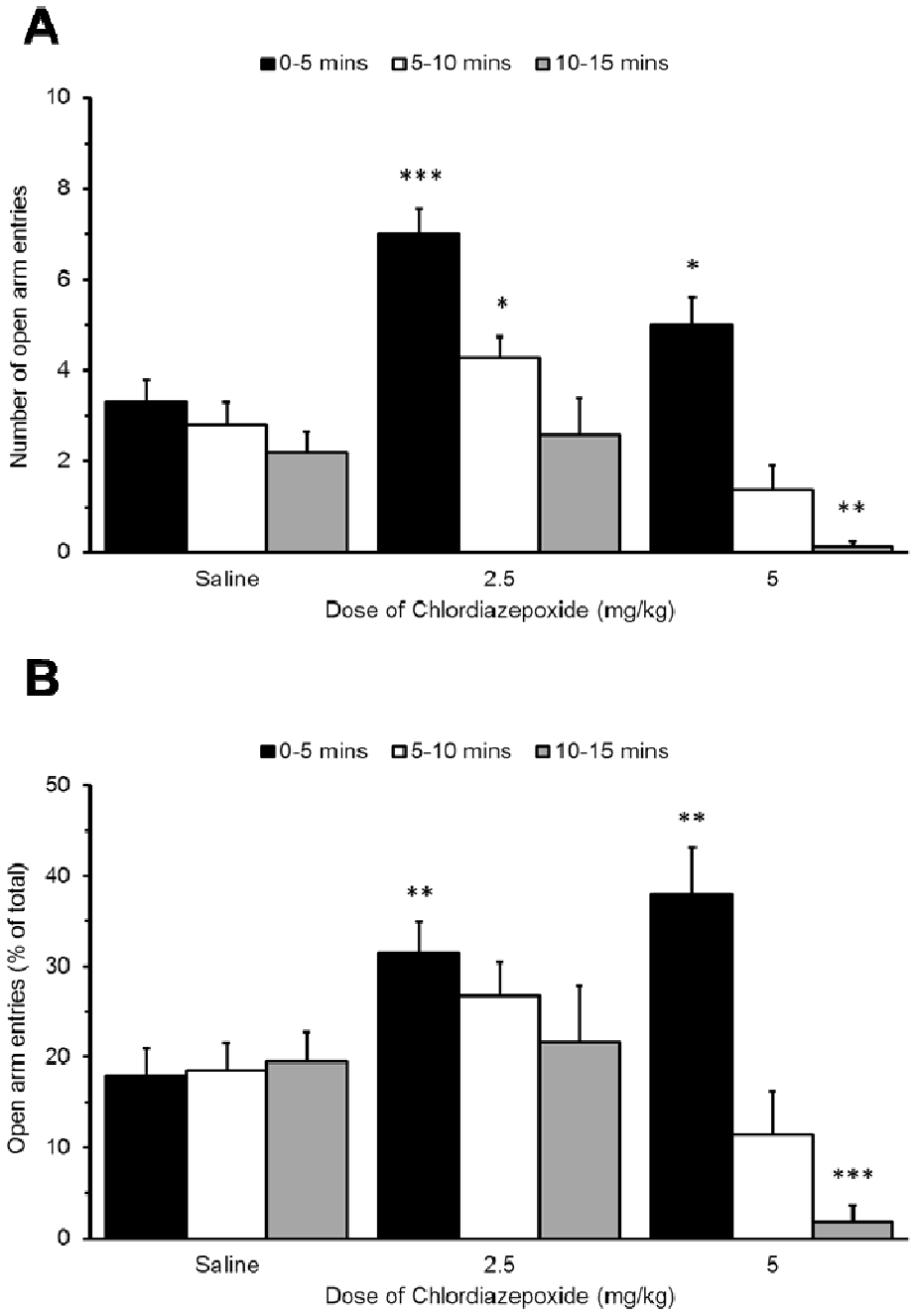

Figure 3.4. – The effect of systemic administration of chlordiazepoxide on the number and percentage of the total entries made into the open arms of the elevated plus-maze. Animals were administered the saline vehicle (*n=10*), 2.5 mg/kg (*n=7*) or 5 mg/kg (*n=8*) chlordiazepoxide i.p. 20 min prior to testing (3 ml/kg). Behaviour on the elevated plus-maze was scored for 15 min (3 x 5 min time bins). **Panel A** shows the total number of entries made into the open runways of the elevated plus-maze; **panel B** expresses these open arm entries as a percentage of the total number of entries made into the open and closed arms of the elevated plus-maze. $p<0.05$, *; $p<0.01$, **; $p<0.001$, *** versus saline control. Data are presented as the mean ± S.E.M.

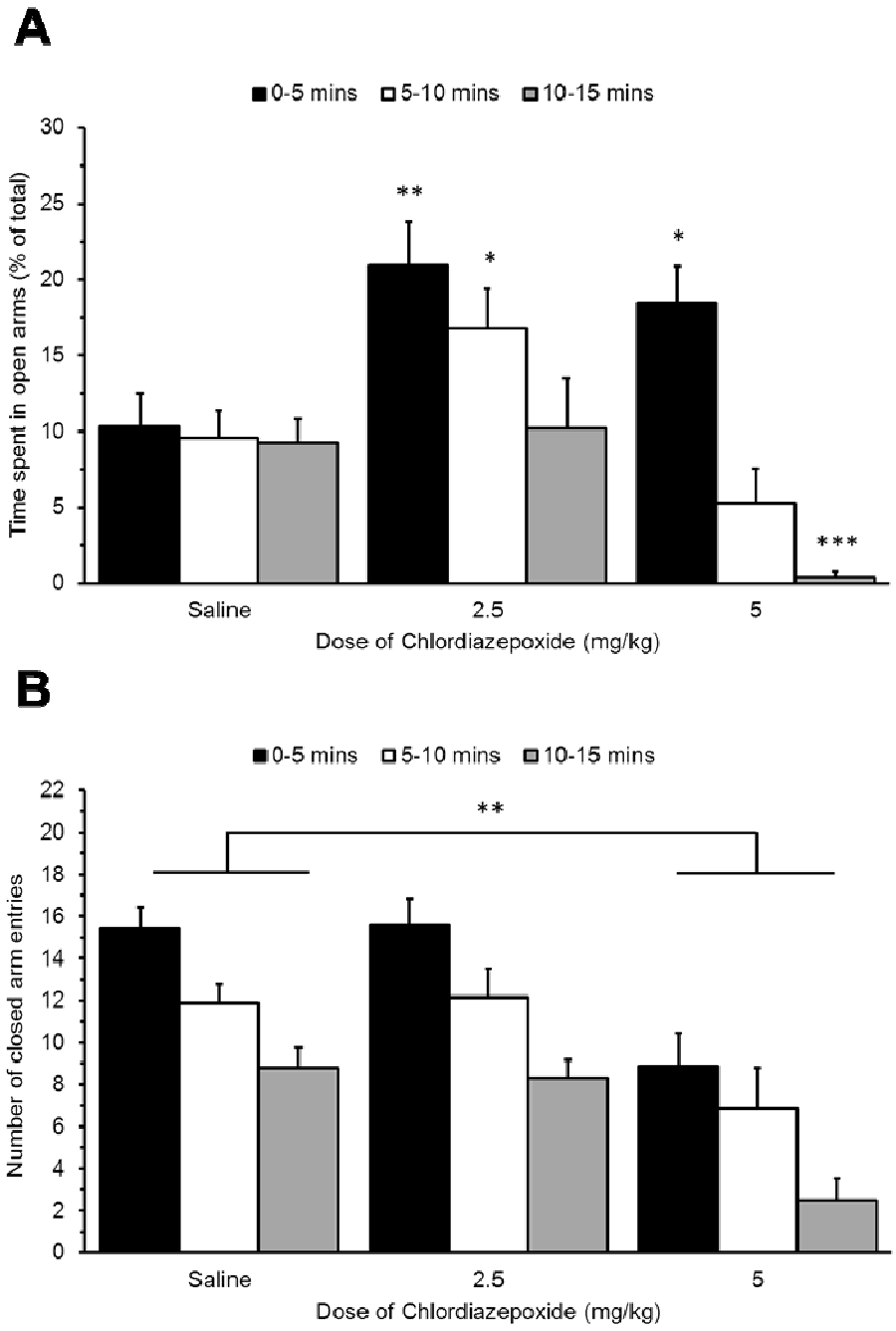

Figure 3.5. – The effect of systemic administration of chlordiazepoxide on the percentage of the total time spent in the open arms and the number of closed arm entries in the elevated plus-maze. Animals were administered the saline vehicle (*n=10*), 2.5 mg/kg (*n=7*) or 5 mg/kg (*n=8*) chlordiazepoxide i.p. 20 min prior to testing (3 ml/kg). Behaviour on the elevated plus-maze was scored for 15 min (3 x 5 min time bins). **Panel A** shows the percentage of the trial time spent exploring the open runways of the elevated plus-maze. **Panel B** expresses the total number of entries made into the enclosed runways of the elevated plus-maze. $p<0.05$, *; $p<0.01$, **; $p<0.001$, *** versus saline control. Data are presented as mean ± S.E.M.

percentage of time spent exploring the open arms (t (16) = -2.660, p<0.05; *figure 3.5.*) in the 0-5 min time bin. However in the 5-10 min time bin, the administration of 5 mg/kg chlordiazepoxide did not significantly increase the number of open arm entries (t (16) = -2.322, p<0.05; *figure 3.4.*), the percentage of the total entries made into the open runways (t (16) = 1.408, n.s.; *figure 3.4.*) or the percentage of the total time spent in the open runways (t (16) = 1.596, n.s.; *figure 3.5.*). In the 10-15 min time bin, the administration of 5 mg/kg chlordiazepoxide significantly reduced the number of entries into the open arms (t (10.585) = 4.773, p<0.01; *figure 3.4.*), the percentage of the total entries made into the open runways (t (16) = 4.680, p<0.001; *figure 3.4.*) and the percentage of the trial time spent exploring the open runways (t (10.143) = 5.862, p<0.001; *figure 3.5.*).

3.2.4. Discussion

Chlordiazepoxide, originally referred to as methaminodiazepoxide, was found to have efficacy as an anxiolytic in clinical trials and was therefore marketed under the trade name Librium in 1960 (Lopez-Munoz *et al*, 2011). Benzodiazepines are positive allosteric modulators of the $GABA_A$ receptor and this is thought to be the mechanism for their anxiolytic effects. Benzodiazepine sensitivity is conveyed by a conserved histidine residue in the N-terminal domain (H101 in the rat α1 subunit) of the α1, α2, α3 and α5 subunit containing receptors (Dunn *et al*, 1999; Wieland *et al*, 1992). However, expression of an arginine at the homologous site of the α6 conveys insensitivity to diazepam (Wieland *et al*, 1992). In agreement, Smith *et al*. (2012) reported that the anxiolytic effects of diazepam were abolished in the α2H101R transgenic mouse.

In agreement with previous studies (Criswell *et al*, 1994; Garcia *et al*, 2011; Pellow *et al*, 1985; Moy *et al*, 1997), the systemic administration of chlordiazepoxide in rats significantly increased the three indices of anxiety-like behaviour in the elevated plus-maze confirming an anxiolytic-like effect. The total number of and percentage of the total arm entries made into the open runways, in addition to the percentage of the trial time spent exploring them, were significantly increased in the initial 5 min of exposure to the plus-maze when the approach-avoidance conflict generated by the novel environment peaks (Montgomery, 1955; Pellow *et al*, 1985). However, the 5 mg/kg dose of chlordiazepoxide significantly decreased the number of entries into the enclosed arms and demonstrates the sedative component of chlordiazepoxide at the higher dose which masks the anxiolytic-like effects. Thus, chlordiazepoxide evoked the anticipated effects on rat behaviour in the elevated plus-maze, data that indicate that the procedure adopted for these studies seems to be a valid measure of anxiety.

3.3. The effect of intra-DRN administration of strychnine on the exploration of the open and closed arms of the elevated plus-maze in animals pre-exposed to an elevated open platform.

3.3.1. Rationale

As previously discussed, the anticipated anxiogenic-like effects of strychnine administered into the DRN may have been masked by the high basal state of anxiety observed in animals exposed to the open field. Previous studies in the laboratory have identified that animals repeatedly exposed to an inescapable elevated open platform stressor show an anxiolytic-like phenotype when subsequently tested in the elevated plus-maze (Storey *et al*, 2006). Although acutely aversive, the repeated exposure to the platform mediates physiological

adaptations in the CNS which appear to reduce the aversive nature of the inescapable platform stressor over a period of 10 days (*section 2.8.1.*; Storey *at al*, 2006; Robertson *et al*, 2005). The aversive components of the elevated open platform and the elevated plus-maze are similar in nature (i.e. an elevated open space). The anxiolytic-like effect of pre-exposure to the platforms is thought to be a consequence of the habituation to an elevated open environment. Therefore, the pre-exposure to the elevated open platforms serves as a behavioural manipulation to reduce the basal levels of the anxiety in the elevated plus-maze. In the lateral amygdala, the anxiolytic-like effect of administration of strychnine or taurine was dependent on the "basal anxiety-state" of the animals in this test (McCool and Chappell, 2007). Therefore in the present study, the effect of intra-DRN administration of strychnine was investigated in animals pre-exposed and naïve to the platform stressor prior to testing in the elevated plus-maze.

3.3.2. Method

Male Sprague-Dawley rats weighing 250-325 g at the beginning of the experiment were implanted with guide cannulae targeted at the DRN (*see section 2.2 and 2.3*). The rats were then divided into two groups. One group (n = 16) was exposed to an inescapable elevated open platform stress (Storey *et al,* 2006; Robertson *et al*, 2005) for 1 hr *per* day for 10 days; the second (n = 20) remained in their home cages throughout. On day 11 post-surgery, animals were transported to the testing room. Under gentle retraint, the stylet was replaced with a 36 ga. needle protruding 2 mm beyond the tip of the cannula into the DRN. Each rat received a microinjection of either strychnine hydrochloride (1 μg), or the aCSF vehicle (500 nl) delivered over 2 min. The needle was left in place for a further 2 min to allow for the diffusion of the

solution from the tip of the neddle. Immediately after the microinjection, the animals were placed at the centre of the elevated plus maze facing an open arm (*see section 2.7*). Behaviour in the elevated plus-maze was recorded on a digital camera for 15 min and scored as 3 x 5 min time bins (number of open arm entries, percentage of total entries made into the open runways, percentage of total time spent in the open runways and the number of entries into the closed arms; *figure 3.6.*). Upon completion of the behavioural study the rats were killed humanly by cervical dislocation and the injection site was verified histologically in fixed sections of the DRN by an observer blind to the treatment the rats had received (*figure 3.6.*). The experiment was performed in groups of 20, 8, 3, 12, 16 and 8 animals. Animals were tested over 17 days, with 4 animals tested in the elevated plus-maze on each day. Of the 67 animals which began the study, 3 animals did not recover upon the termination of anaesthesia. In addition, a further 2 animals were removed prior to behavioural testing as one animal developed a post-operative infection and a second animal damaged the cement cap post-surgery. On the testing day, an additional 7 animals could not be administered the experimental solution due to the blocking of the guide cannula. Furthermore, 15 animals were removed from the study as the histological investigation identified administrations outwith the DRN. An additional 4 animals were removed from the final analysis as they were identified as statistical outliers (i.e. greater than double the standard deviation from the mean). The behaviour was analysed by a repeated measures ANOVA using pre-treatment (stress or home cage) and treatment (aCSF or strychnine) as the between-subjects factors analysed and time as the within-subjects factor. *Post-hoc* independent samples t-tests are reported for the investigation of interactions between the within and between subject factors.

3.3.3. Results

The number of closed arm entries, a measure of locomotor activity, was significantly increased in the initial 0-5 min time bin by the pre-exposure of the animals to the elevated open platforms. Exposure to the elevated open platforms significantly increased both the number and percentage of entries into the open runways and the percentage of the trial time spent in the open runways when compared to the home cage control suggesting an anxiolytic-like effect. Furthermore, the effect of pre-exposure to the platforms on the percentage of the trial time spent in the open runways varied between the time bins suggesting that animals exposed to the platforms habituated to the elevated plus-maze differently from animals remaining in the home cage during the trial. Additionally, the intra-DRN administration of strychnine partially reversed the platform-induced increase in the percentage of the trial time spent in the open runways. However, the administration of strychnine had no significant influence on the number of closed arm entries nor the number and percentage of entries made into the open runways. Therefore, in animals pre-exposed to the elevated platforms the administration of strychnine decreases the latency of escape from the open runways of the elevated plus-maze.

Repeated measures ANOVA showed that prior exposure to the elevated open platform significantly increased the number of open arm entries ($F(1,32)=49.033$; $p<0.001$; *figure 3.7.*), the percentage of the total arm entries made into the open runways ($F(1,32) = 33.679$; $p<0.001$; *figure 3.7.*), the percentage of the total time spent in the open runways ($F(1,32) = 65.917$; $p<0.001$; *figure 3.8.*) and the number of the closed arm entries ($F(1,32) = 6.009$; $p<0.05$; *figure 3.8.*). The intra-DRN administration of strychnine significantly reduced the percentage of the total time spent in the open arms ($F(1,32) =$

9.027; p<0.01; *figure 3.8.*). However, there was no significant effect of strychnine on the number of open arm entries ($F(1,32)=2.105$; n.s.; *figure 3.7.*), the percentage of entries made into the open arms ($F(1,32) = 2.436$; n.s.; *figure 3.7.*) or the number of closed arm entries ($F(1,32) = 0.010$; n.s.; *figure 3.8.*). A significant interaction between the repeated exposure to the elevated platforms and the administration on the strychnine influenced the percentage of the total time spent exploring the open runways (*pretreatment x treatment*; $F(1,32) = 10.774$; p<0.01; *figure 3.8.*). However, there was no such interaction influencing the number of open arm entries (*pretreatment x treatment*; $F(1,32)=2.105$; n.s.; *figure 3.7.*), the percentage of total entries made into the open runways (*pretreatment x treatment*; $F(1,32) = 0.647$; n.s.; *figure 3.7.*) or on the number of closed arm entries (*pretreatment x treatment*; $F(1,32) = 0.301$; n.s.; *figure 3.8.*). *Post hoc* analysis showed in rats repeatedly exposed to the elevated open platform stressor ($t\,(14) = 3.381$; p<0.001; *figure 3.8.*), but not those remaining in the home cage ($t\,(18) = -0.285$; n.s.; *figure 3.8.*), the intra-DRN administration of strychnine significantly reduced the percentage of the trial time spent exploring the open runways.

The number of open arm entries ($F(2,64)=4.904$; p<0.05), the percentage of the total entries made into the open runways ($F(2,64) = 4.426$; p<0.05), the percentage of the total time spent exploring the open runways ($F(2,64) = 5.841$;p<0.01) and the number of closed arm entries ($F(2,64) = 14.106$; p<0.001) varied significantly across the time bins.

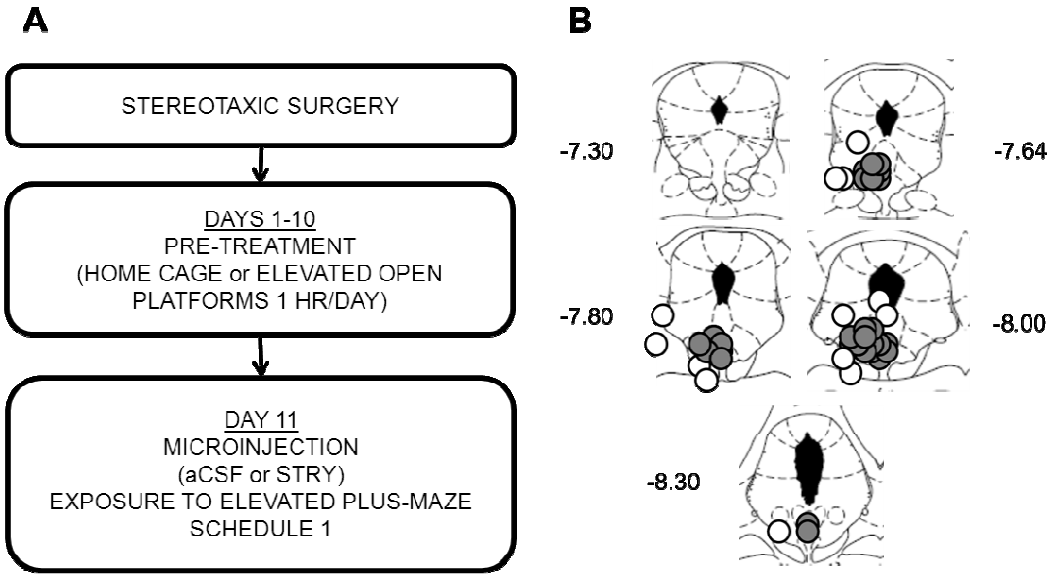

Figure 3.6. – Experimental design and a representative schematic showing the distribution of microinjection sites targeted at the DRN. Panel A shows the experimental design, where the number of days denotes the number of days post-surgery. **Panel B** is a diagramatic representation of the DRN and surrounding area adapted from the coronal sections of Paxinos and Watson (1998). Numerical values indicate the posterior distance from Bregma. Following behavioural testing and cervical dislocation, 2% Chicago sky blue dye was administered into the DRN. The brain was removed and fixed in a 4% formalin solution for 48 hr and then transferred to a 30% sucrose solution for 72 hr. Coronal sections (36 µm) containing the DRN were mounted on gelatinised slides and stained using cresyl violet to visualise the topography of the brain. Filled circles show examples of cannulations which resulted in the successful administration of substances into the DRN and were included in the study. Open circles depict the location of injection sites excluded from the study either due to the administration of substances outside of the DRN or by the occlusion of the cerebral aqueduct. Histological assessment of the cannulations was performed by an observer blind to the behavioural and treatment history of the animal.

The effect of pre-exposure to the elevated open platform on the percentage of the total time spent in the open runways (*time x pretreatment*; $F(2,64) = 3.952$; $p<0.05$) and the number of closed arm entries (*time x pretreatment*; $F(2,64) = 6.526$; $p<0.01$) was significantly different between the time bins. However, the effect of the platform stress on the number (*time x pretreatment*; $F(2,64)=2.309$; n.s.) or the percentage of total entries made into the open runways (*time x*

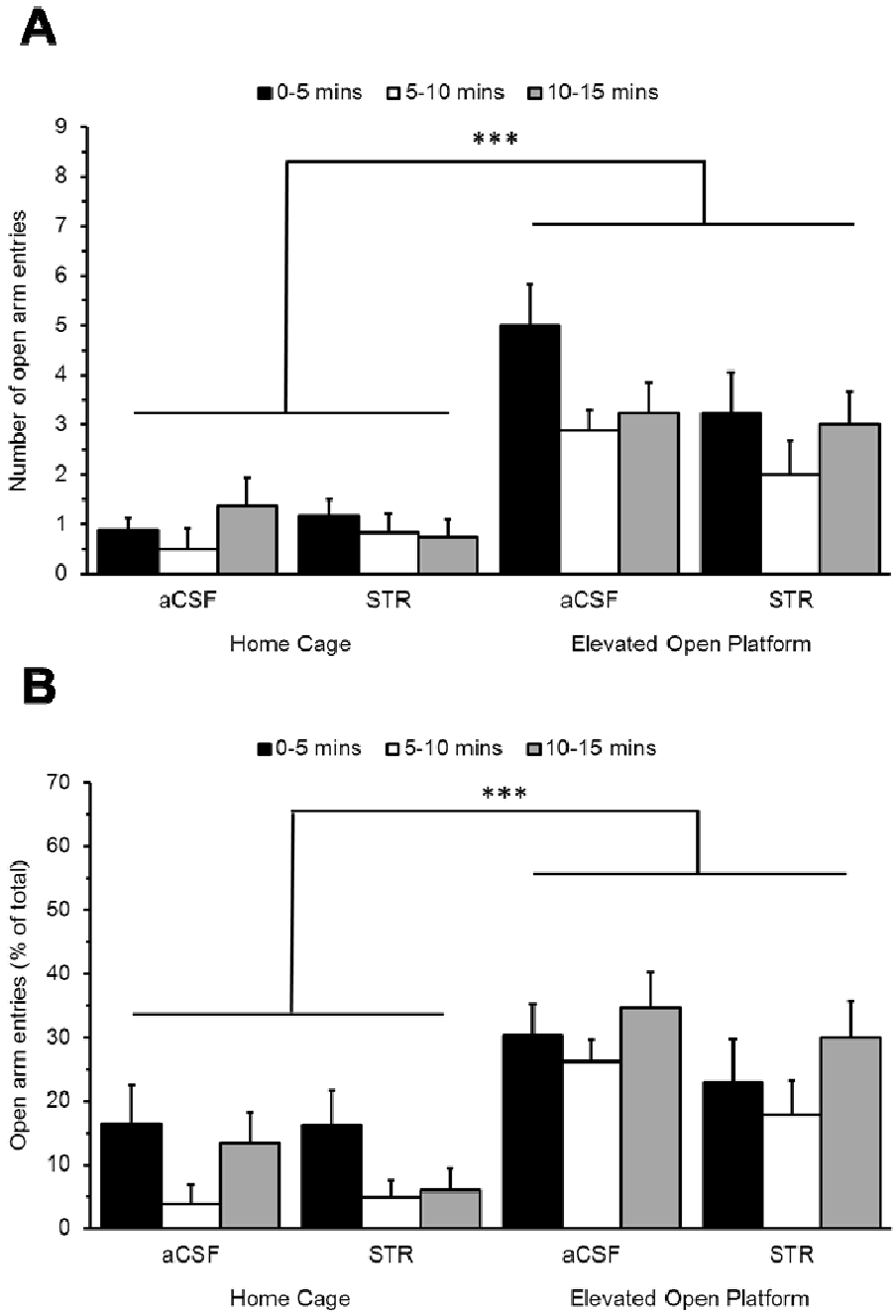

Figure 3.7. – The effect of intra-DRN administration of strychnine on the number and percentage of total arm entries made into the open arms of the elevated plus-maze in animals repeatedly exposed to an elevated open platform stressor. Animals were exposed to an elevated open platform stressor (*n=16*) for 1h daily over 10 consecutive days or remained in the home cage throughout (*n=20*). On day 11 post-surgery, animals were administered 1 µg strychnine hydrochloride (*n=20*) or the aCSF vehicle (500 nl; *n=16*) into the DRN via the indwelling cannulae prior to a 15 min exposure to the elevated plus-maze. Animals repeatedly exposed to the elevated open platform, but not those remaining in the home cage, showed an increase in both the number (**panel A**) and percentage of total arm entries made into the open runways during the 15 min trial (**panel B**). $p<0.001$, *** versus home cage control. Data are presented as mean ± S.E.M.

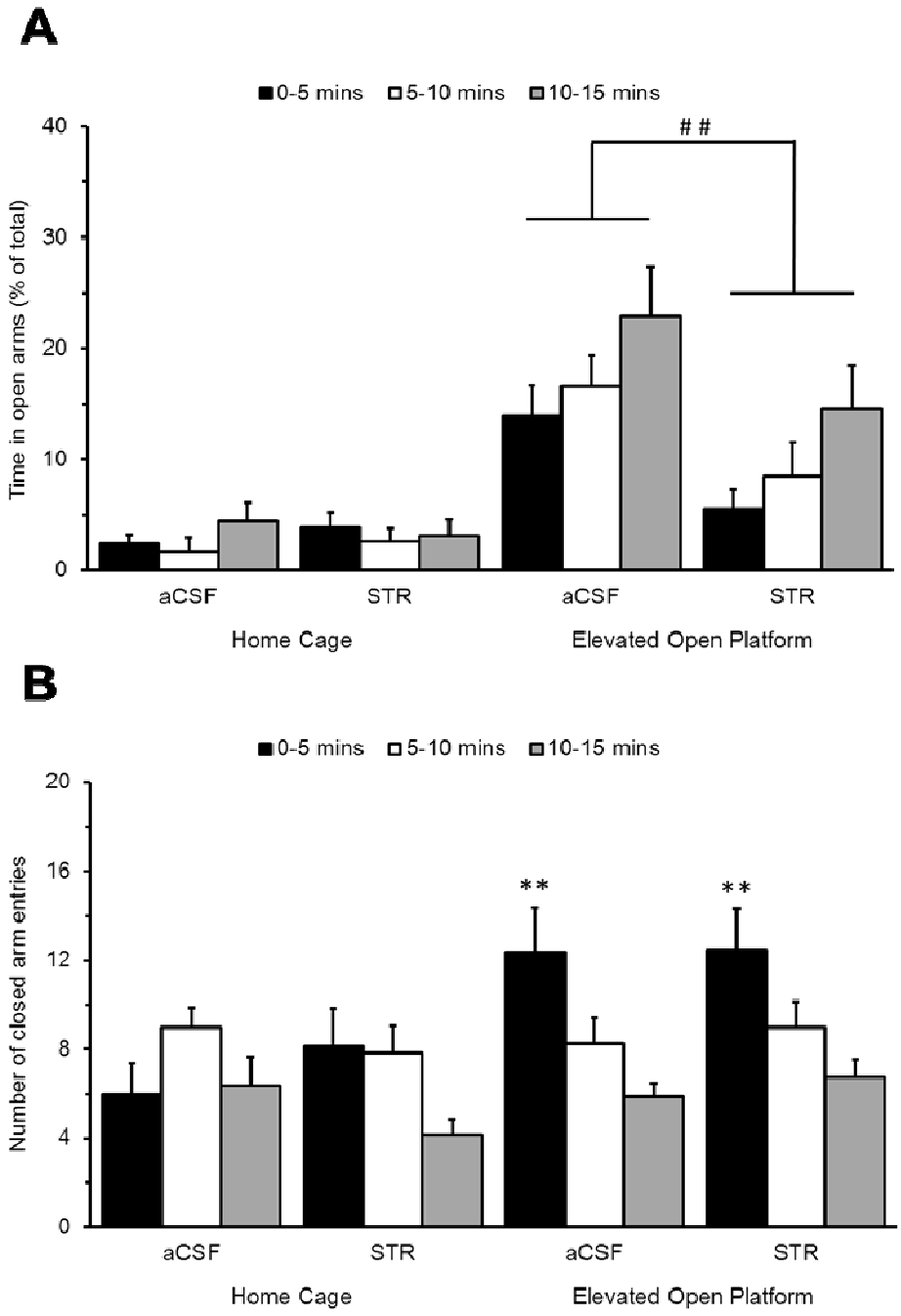

Figure 3.8. – The effect of intra-DRN administration of strychnine on the percentage of the total time spent exploring the open runways and the number of entries into the enclosed arms of the elevated plus-maze in animals repeatedly exposed to an elevated open platform stressor. Animals were exposed to an elevated open platform stressor (*n=16*) for 1h daily over 10 consecutive days or remained in the home cage throughout (*n=20*). On day 11 post-surgery, animals were administered 1 µg strychnine hydrochloride (*n=20*) or the aCSF vehicle (500 nl; *n=16*) into the DRN via the indwelling cannulae prior to a 15 min exposure to the elevated plus-maze. Animals repeatedly exposed to the elevated open platform showed a significant increase in the time spent in the open runways during the 0-5 min, 5-10 min and 10-15 min time bins. Intra-DRN administration of strychnine selectively reversed the increase in the

percentage of time spent in the open runways of animals exposed to the elevated platforms (**panel A**). The number of closed arm entries in the initial 0-5 min time bin was significantly increased in animals repeatedly exposed to the elevated open platform (**panel B**). $p<0.01$, ** versus home cage control; $p<0.01$,$^{\#\#}$ versus aCSF control. Data are presented as mean ± S.E.M.

pretreatment; $F(2,64) = 1.933$; n.s.) did not significantly differ between the time bins. The number of open arm entries (*time x treatment*; $F(2,64)=0.253$; n.s.), the percentage of the total entries made into the open arms (*time x treatment*; $F(2,64) = 0.93$; n.s.), the percentage of the trial time spent exploring the open runways (*time x treatment*; $F(2,64) = 0.126$; n.s.) and the number of entries into the enclosed arms (*time x treatment*; $F(2,64) = 0.760$; n.s.) were not significantly influenced by an interaction between the administration of strychnine and the time bins. The number of open arm entries (*time x pretreatment x treatment*; $F(2,64)=1.771$; n.s.), the percentage of the total arm entries made into the open runways (*time x pretreatment x treatment*; $F(2,64) = 0.555$; n.s.), the time spent exploring the open runways (*time x pretreatment x treatment*; $F(2,64) = 0.128$; n.s.) and the number of entries into the enclosed runways (*time x pretreatment x treatment*; $F(2,64) = 1.543$; n.s.) were not significantly influenced by an interaction between the pre-exposure to the platforms and the administration of strychnine over the time bins.

Post-hoc analysis showed that the elevated open platforms significantly increased the percentage of the trial time spent in the open arms during the 0-5 min ($t(20.985) = -3.250$, $p<0.01$; *figure 3.8.*), the 5-10 min ($t(20.985) = -4.523$, $p<0.001$; *figure 3.8.*) and 10-15 min time bins ($t(18.865) = -4.949$, $p<0.001$; *figure 3.8.*). In addition, pre-exposure to the elevated open platforms significantly increased the number of closed arm entries in the 0-5 min time bin

(t (34) = -3.134, p<0.01; *figure 3.8.*), but had no significant effects on the 5-10 min bin (t (34) = -0.297, *n.s.*; *figure 3.8.*) or the final 5 minute bin (t (34) = -1.506, *n.s.*; *figure 3.8.*).

3.3.4. Discussion

In agreement with the findings in the open field, animals naïve to the elevated open platform stressor showed high basal levels of anxiety when tested in the elevated plus-maze. In addition, the intra-DRN administration of strychnine did not have any significant effects on the indices of anxiety or the locomotor activity of these animals. Therefore, as was observed in the open field, the effects of strychnine in the elevated plus-maze may be masked in elevated open platform-naïve animals.

In agreement with previous findings in the laboratory, repeated exposure to the inescapable elevated open platform stressor reduced anxiety-like behaviours in animals tested on the elevated plus-maze characteristic of an anxiolytic-like effect (Storey *et al*, 2006; Robertson *et al*, 2005). In animals exposed to the elevated open platform stressor, but not those remaining in the home cage, the intra-DRN administration of strychnine decreased the latency of escape from the open runways of the elevated plus-maze suggesting the reversal of a selective component of the anxiolytic-like effects induced by the exposure to the elevated open platforms. This partial reversal of the anxiolytic-like effect of the platforms was independent of any changes in the locomotor activity of the animals and therefore cannot be attributed to a stimulation of motor activity. Gonzalez and File (1997) have reported that the intra-DRN administration of the benzodiazepine midazolam in plus-maze naive rats increased the percentage of time spent in the open runways. Therefore, the latency of escape from the

aversive environment may, in part, be mediated by the serotonergic neurons in the DRN.

Graeff *et al.* (1996) anticipated that drugs which increase the firing of serotonergic neurons in the DRN would facilitate inhibitory avoidance of the open runways (i.e. remain in the closed runways) and impair one-way escape from the aversive environment (i.e. escape from the open arms) in an elevated t-maze. Inhibitory avoidance is measured by placing the animal in the closed arm of the t-maze and measuring the latency to exit the arm. This is considered a measure of learned fear and is thought to be mediated by serotonergic transmission to the amygdala and frontal cortex which mediate active escape or avoidance behaviours in response to perceived or distal threats (Graeff *et al*, 1996). Similarly, the one-way escape is measured by placing the animal in the open 'aversive' arm and measuring the latency to escape to the enclosed arm. This is considered a measure of innate fear, and is mediated by the serotonergic projections to the periaqueductal grey which inhibit fight/flight responses to immediate or proximal threats (Graeff *et al*, 1996). In agreement, Graeff *et al*. (1996) reported the presumed excitation of the serotonergic neurons elicited by the intra-DRN administration of a subtoxic dose of kainic acid significantly increased inhibitory avoidance of the open runways and increased the latency of one-way escape from the open runways. The administration of 8-OH DPAT into the DRN was found to decrease inhibitory avoidance but had no effect on one-way escape (Graeff *et al*, 1996). Therefore, previous studies in the t-maze would appear to contradict the findings of the present study. An increase in the escape from the open runways of the t-maze is characteristic of an increase in panic-like behaviours, which are thought to be mediated by the inhibition, as opposed to the facilitation, of the excitation of the

serotonergic neurons projecting from the DRN to the periaqueductal grey (Graeff and Rawlins, 1980).

However, it is important to note that in the modified t-maze the animal is placed within the confines of the aversive environment, forcing confrontation with the stressor, whereas in the elevated plus-maze the animal is placed in the centre of the maze and thus explores the aversive open runways voluntarily. In addition, the open runways of the elevated plus-maze are considered aversive due to the perception of a potential threat. Conversely, in the t-maze the experimenter may serve as a proximal threat when measuring one-way escape. Therefore, the latency to escape from the open runways of the elevated plus-maze and the t-maze may be mediated by distinct neuronal substrates (*section 1.1.5.*). The increased escape from the open runways, observed with the intra-DRN administration of strychnine in animals pre-exposed to the elevated platforms, may be interpreted as an increase in learned active avoidance behaviours, as opposed to an increase in panic-like behaviours, in response to the perceived threat posed by the open runways of the plus-maze.

When confronted with a stressor, the animal responds with either active or passive coping mechanisms which are dependent on the nature of the stressor (Blanchard *et al*, 2011). The absence of behavioural escape from the elevated open platform stressor induces physiological coping mechanisms which are, in part, mediated by alterations in the serotonergic system (*section 2.8.1*.). Using in vivo microdialysis, Bland *et al*. (2003) reported a prolonged increase in serotonin overflow in the vmPFC of animals acutely exposed to inescapable stress. However, Storey *et al*. (2006) reported that repeated exposure to the inescapable platform stressor abolishes the increase in serotonin overflow in the frontal cortex and facilitates serotonin overflow in the dorsal hippocampus.

Therefore, previous studies have identified that the habituation to the inescapable platform stressor results in the inhibition of the serotonergic neurons of the DRN and facilitates the excitation of the serotonergic neurons of the MRN. The present study suggests that the inhibition of the serotonergic neurons of the DRN in response to the habituated stressor may be mediated by the activation of the strychnine-sensitive glycine receptors. However, this interpretation should considered parsimoniously as platform naïve animals show a significantly lower number of open arm entries when compared with animals pre-exposed to the elevated open platform. Therefore the number of entries into the open runways, which is independent of the administration of strychnine in both the animals pre-exposed to the elevated open platform and those naïve to the stressor, may mask an effect of strychnine on the time spent in the open runways in the platform-naïve animals.

3.4. The effect of intra-DRN administration of glycine on the exploration of the open and closed arms of the elevated plus-maze.

3.4.1. Rationale

Investigations in the open field and elevated plus-maze suggested that intra-DRN administration of strychnine increases anxiety-like behaviours in the animals with a low basal state of anxiety. Therefore the hypothesis was that activation, as opposed to the blockade, of the receptors would induce an anxiolytic-like effect in animals with a high basal state of anxiety in a test of anxiety. A study was performed to investigate the putative anxiolytic-like effects of intra-DRN administration of the agonist glycine in the elevated plus-maze test of anxiety.

3.4.2. Method

Male Sprague-Dawley rats weighing 250-300 g at the beginning of the experiment were implanted with guide cannulae targeted at the DRN (*see section 2.2 and 2.3*). The rats were left for 10 days in the home cage to recover from the surgery. On day 11 post-surgery, animals were transported to the testing room. Under gentle retraint, the stylet was replaced with a 36 ga. needle protruding 2 mm beyond the tip of the cannula into the DRN. The rats were administered the aCSF vehicle (500 nl; n=4), 1 ng (n=3), 11 ng (n=4) or 112 ng glycine (n=3) over 2 mins by means of the needle inserted through the guide cannula. The needle was left in place for a further 2 min to allow for diffusion of the solution from the tip of the needle. The animals were then immediately placed at the centre of the elevated plus-maze facing an open arm (*see section 2.7*). Behaviour in the elevated plus-maze was recorded on a digital camera for 15 min and scored as 3 x 5 min time bins (number of open arm entries, percentage of total entries made into the open runways, percentage of total time spent in the open runways and the number of entries into the closed arms; *figure 3.9.*). Upon completion of the behavioural study the rats were killed humanly by cervical dislocation and the injection site was verified histologically in fixed sections of the DRN by an observer blind to the treatment the rats had received (*figure 3.9.*). The experiment was performed using a single batch of 16 animals. Animals were tested over 4 days, with 4 animals tested in the elevated plus-maze on each day. Of the 16 animals which began the study, 2 animals could not be administered the experimental solution due to the blockage of the guide cannula. The behaviour was analysed by a repeated measures ANOVA using treatment (glycine or aCSF) as the independent factor analysed and time as the within-subjects factor. Greenhouse-Geisser correction factor applied to data which did not adhere to Mauchly's test of sphericity.

3.4.3. Results

In vitro, the application of exogenous glycine reduces the neuronal excitation of the serotonergic neurons of the DRN via the activation of the strychnine-sensitive glycine receptors (Maguire *et al*, 2013). Therefore, the intra-DRN administration of glycine *in vivo* was hypothesised to induce an anxiolytic-like phenotype in animals exposed to the elevated plus-maze. However, the intra-DRN administration of glycine had no significant effect on either the indices of anxiety (number and percentage of total arm entries made into the open runway and the percentage of the trial time spent in the open runways) or locomotor activity (the number of enclosed arm entries).

The intra-DRN administration of glycine did not significantly influence the number of open arm entries ($F(3,10)=1.286$; n.s.; *figure 3.10.*), the percentage of the total arm entries made into the open runways ($F(3,10)=1.830$; n.s.; *figure 3.10.*), the percentage of the total time spent in the open arms ($F(3,10)=1.934$; n.s. *figure 3.11.*) or the number of closed arm entries ($F(3,10)=2.680$; n.s.; *figure 3.11.*).

The number of open arm entries ($F(2,20)=6.254$; $p<0.01$) and the number of closed arm entries ($F(2,20)=13.657$; $p<0.001$) differed significantly between the time bins. However, the percentage of the total arm entries made into the open runways ($F(2,20)=2.839$; n.s.) and the percentage of the total time spent exploring the open runways ($F(2,20)=2.752$; n.s.) were not significantly different between the time bins.

There was no significant interaction between the administration of glycine and the time bins on the number of open arm entries (*time x treatment*; $F(6,20)=0.491$; n.s.; *figure 3.10.*), the percentage of entries made into the open

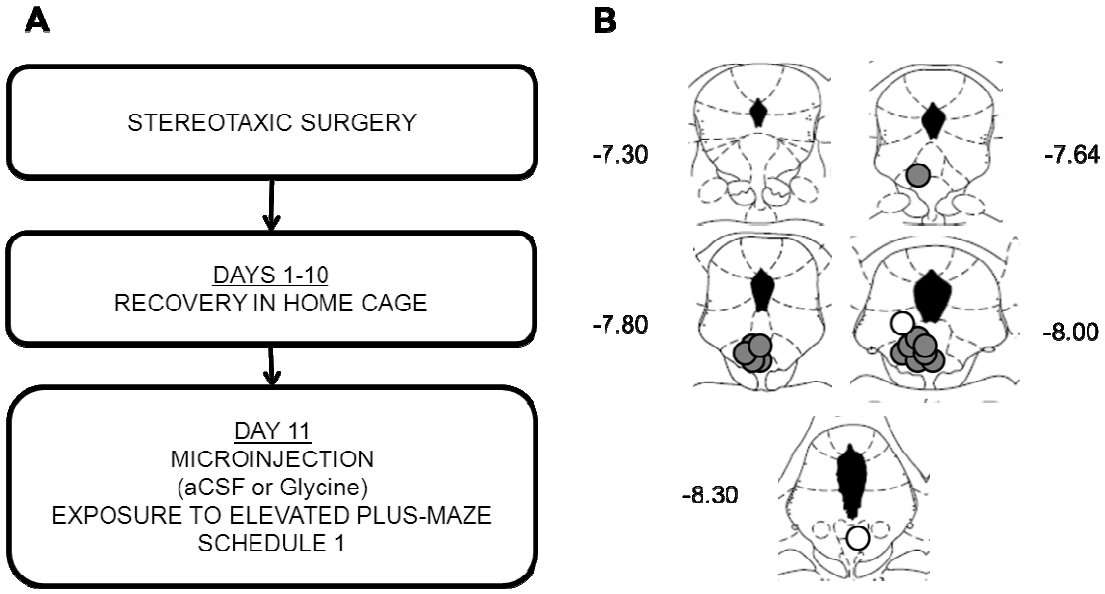

Figure 3.9. – Experimental design and a representative schematic showing the distribution of microinjection sites targeted at the DRN. Panel A shows the experimental design, where the number of days denotes the number of days post-surgery. **Panel B** is a diagramatic representation of the DRN and surrounding area adapted from the coronal sections of Paxinos and Watson (1998). Numerical values indicate the posterior distance from Bregma. Following behavioural testing and cervical dislocation, 2% Chicago sky blue dye was administered into the DRN. The brain was removed and fixed in a 4% formalin solution for 48 hr and then transferred to a 30% sucrose solution for 72 hr. Coronal sections (36 µm) containing the DRN were mounted on gelatinised slides and stained using cresyl violet to visualise the topography of the brain. Filled circles show examples of cannulations which resulted in the successful administration of substances into the DRN and were included in the study. Open circles depict the location of injection sites excluded from the study either due to the administration of substances outside of the DRN or by the occlusion of the cerebral aqueduct. Histological assessment of the cannulations was performed by an observer blind to the treatment and behavioural history of the animals.

runways (*time x treatment*; F(6,20)=0.268; n.s.; *figure 3.10.*), the percentage of time spent exploring the open runways (*time x treatment*; *F*(6,20)=0.632; n.s.; *figure 3.11.*) and the number of closed arm entries (*time x treatment*; *F*(6,20)=0.749; n.s.; *figure 3.11.*).

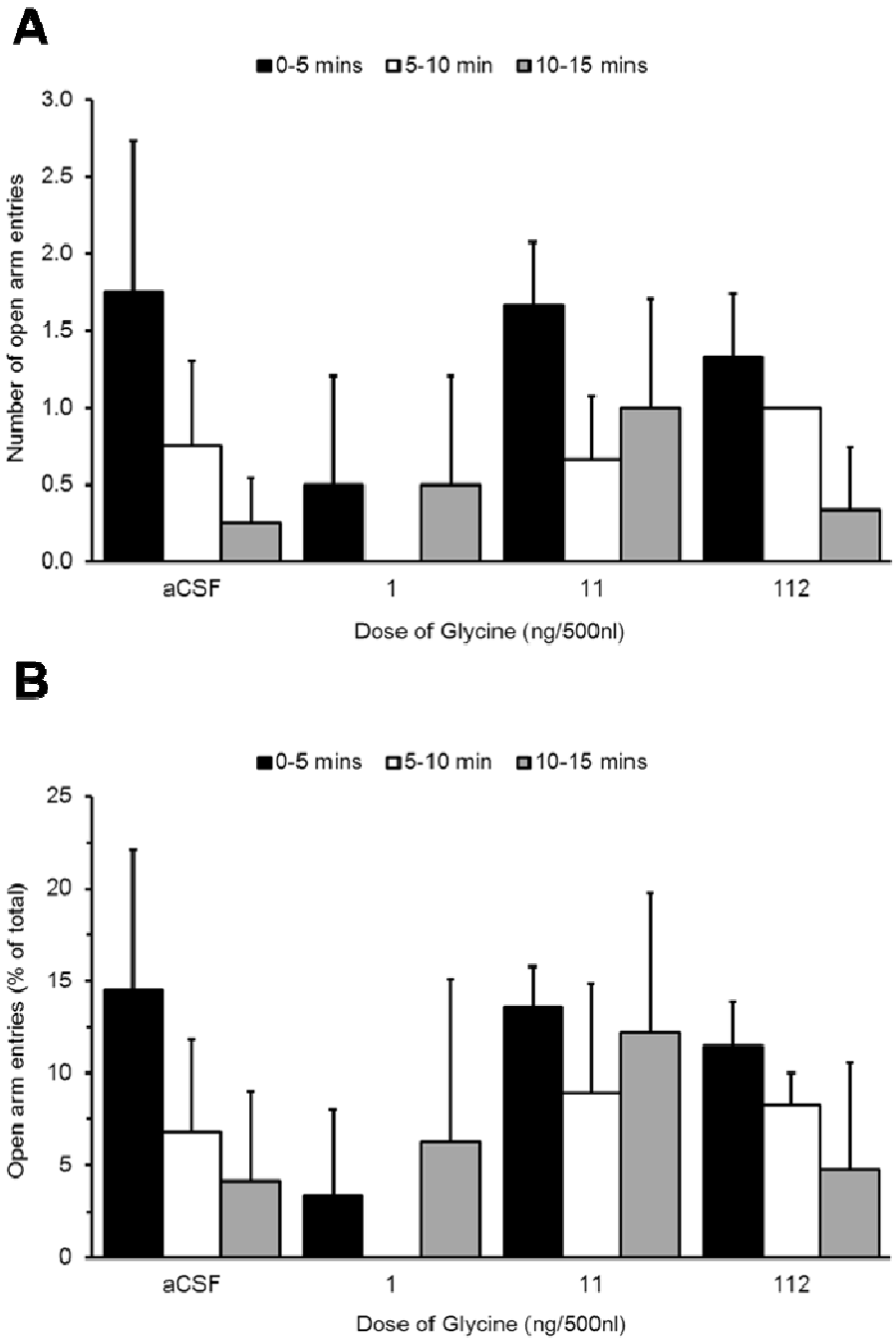

Figure 3.10. – The effect of intra-DRN administration of glycine on the number and percentage of the total entries made into the open runways of the elevated plus-maze. Following surgery, animals remained in the home cage for 10 days to recover. On day 11 post-surgery, animals were administered 1 ng (*n=3*), 11 ng (*n=4*) or 112 ng glycine (*n=3*) or the aCSF vehicle (500 nl; *n=4*) into the DRN via indwelling cannulae prior to a 15 min exposure to the elevated plus-maze. **Panel A** shows that the administration of glycine did not significantly influence the number of entries made into the open runways of the elevated plus-maze. **Panel B** shows that the intra-DRN administration of glycine had no significant influence on the percentage of the total entries made into the open runways. Data are presented as the mean ± S.E.M.

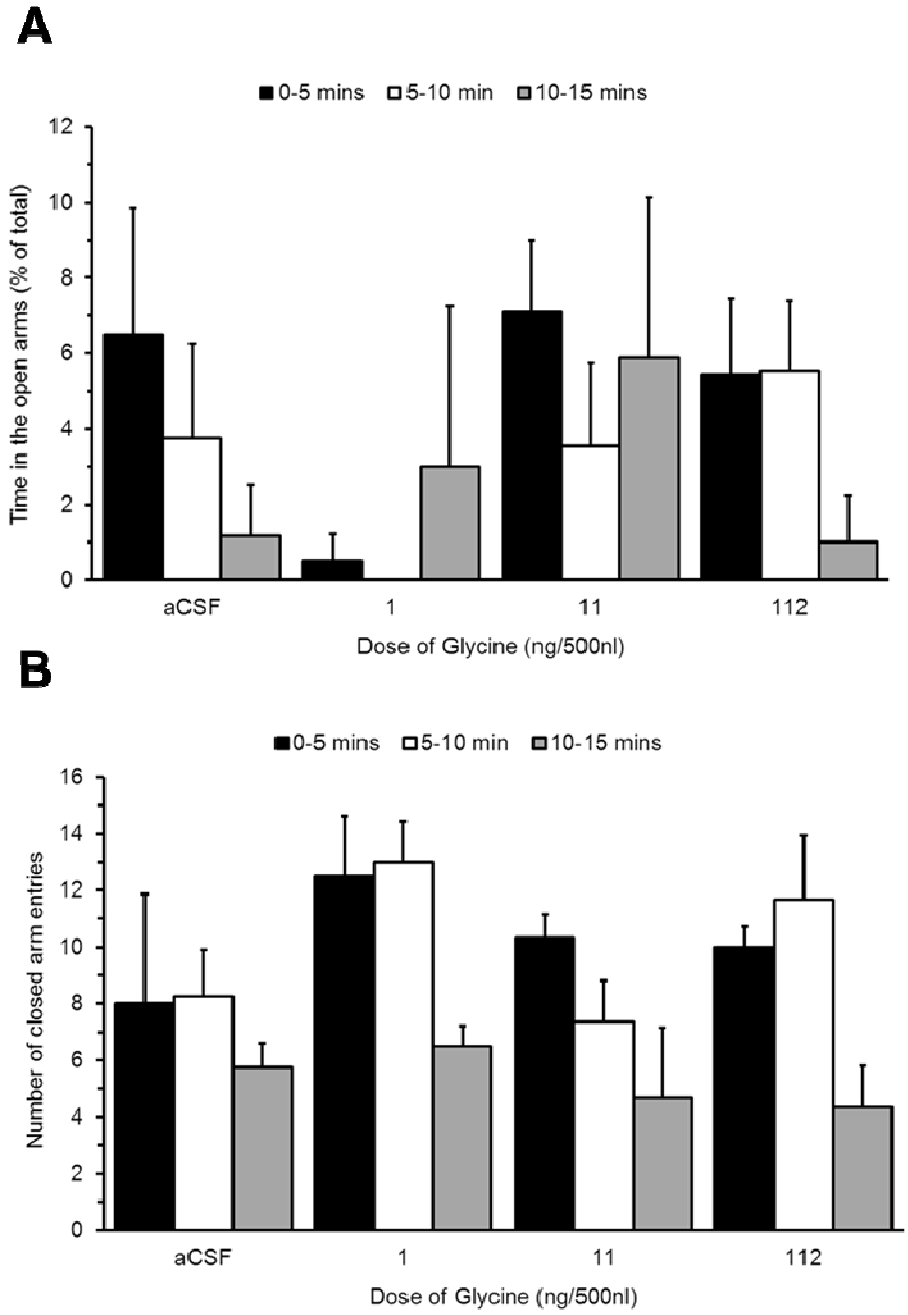

Figure 3.11. – The effect of intra-DRN administration of glycine on the time spent exploring the open runways and the number of entries into the closed arms of the elevated plus-maze. Following surgery, animals remained in the home cage for 10 days to recover. On day 11 post-surgery, animals were administered 1 ng (*n=3*), 11 ng (*n=4*) or 112 ng glycine (*n=3*) or the aCSF vehicle (500 nl; *n=4*) into the DRN via indwelling cannulae prior to a 15 min exposure to the elevated plus-maze. **Panel A** shows that the percentage of the total time spent exploring the open runways of the elevated plus-maze was not significantly influenced by the administration of glycine into the DRN. **Panel B** shows that the number of entries made into the enclosed arms of the maze was not significantly influenced by the administration of glycine. Data are presented as the mean ± S.E.M.

3.4.4. Discussion

Statistically, the results suggest that the local administration of glycine into the DRN has no effect on the indices of anxiety-related behaviours (number of open arm entries, percentage of the total entries made into the open arm or the percentage of the total time spent in the open runways) or locomotor activity (number of enclosed arm entries) as measured in the elevated plus-maze. Based upon the data generated at present, a power analysis suggests that a sample size of 6 animals in each group would be required to validate these findings and therefore the present study is underpowered.

However, the administration of 1 ng glycine is trending towards an anxiogenic-like effect, as opposed to the anxiolytic-like effect as was hypothesised. It is anticipated that the administration of glycine into the DRN would activate the strychnine-sensitive glycine receptors therefore depressing the neuronal excitation of the serotonergic neurons and induce an anxiolytic-like effect in the elevated plus-maze. However, a potential anxiogenic-like effect may potentially be mediated if the intra-DRN administration of 1 ng glycine facilitates the NMDA-mediated excitation of the serotonergic neurons of the DRN (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988). In agreement, the administration of the GlyT1 inhibitors Org-24461 and NFPS was reported to increase the excitation of the serotonergic neurons in the DRN (Papp *et al*, 2008). However, Keck *et al*. (2008) demonstrated that the activation of extrasynaptic strychnine-sensitive glycine receptors in the hippocampus results in an increased conductance which results in an inhibitory ‘shunt’ of excitatory innervations.

Furthermore, the experimental findings of the present study may be confounded due to the uptake of the exogenous glycine mediated by the GlyT1 and GlyT2 transporters (section 1.5.3). Therefore, the administration of higher doses of glycine may be required to elicit a behavioural response. In agreement, Schmitt *et al*. (1995) demonstrated that the administration 3 µg - 6 µg glycine into the PAG induces anxiogenic-like effects in the elevated plus-maze. However, the administration of higher doses may exert non-specific osmotic effects upon the neuronal tissue of the target site (Greenshaw, 1986). Therefore, an alternative approach which may be included in the study would be to co-administer glycine in conjecture with an inhibitor of glycine transporters such as sarcosine and ALX5407.

4. Are components of the anxiolytic properties of ethanol mediated by the potentiation of the strychnine-sensitive glycine receptors in the DRN?

4.1. The effect of systemic administration of ethanol on the exploration of the open and closed arms of the elevated plus-maze.

4.1.1. Rationale

Previous studies have shown that the systemic administration of ethanol significantly reduces anxiety-like behaviours in the elevated plus-maze (Criswell *et al*, 1994; Moy *et al*, 1997; Prunell *et al*, 1994a; Prunell *et al*, 1994b; Langen *et al*, 2002). In addition, the anxiolytic properties of systemically administered ethanol have also been reported in the social interaction test (File *et al*, 1976), the Geller-Seifter test (Aston-Jones *et al*, 1984) and the open field (Da Silva *et al*, 2005). Therefore, the present study aimed to investigate the reported anxiolytic-like effects of ethanol administered (i.p.) in rats exposed to the elevated plus-maze.

4.1.2. Method

Male Sprague-Dawley rats (250-300g) were housed in cages of four throughout. Animals received daily i.p. injections of saline (1 ml/kg) for 7 consecutive days in the testing room in order to habituate to the injection procedure. On day 8, animals received an i.p. injection of 0.5 g/kg (n=4), 0.75 g/kg (n=4), 1 g/kg (n=8) or 1.5 g/kg ethanol (n=8) or the saline vehicle (6 ml/kg; n=8) 20 minutes prior to exposure to the elevated plus-maze. Animals were placed in the centre of the maze facing an open runway (*see section 2.7*). Behaviour was recorded for 15 mins using a digital camera and scored as 3 x 5 min time bins (number of open arm entries, percentage of total entries made into the open runways, percentage of total time spent in the open runways and the number of entries into the closed arms; *figure 4.1. and figure 4.2*). The experiment was performed using two batches of 16 animals. Animals were tested over 8 days, with 4

animals tested in the elevated plus-maze on each day. Of the 32 animals which began the study, one animal was identified as a statistical outlier (i.e. double the standard deviation from the mean) and a further animal was excluded as the administration may have been into the bladder and not the peritoneum. Data were analysed by repeated measures ANOVA with dose of ethanol as the between-subjects factor and time as the within-subjects factor. *Post-hoc* analysis of the between-subject factor was performed using the Games-Howell test. Greenhouse-Geisser correction factor applied to data which did not adhere to Mauchly's test of sphericity.

4.1.3. Results

The i.p. administration of ethanol had no significant influence on any of the three indices of anxiety in the elevated plus-maze. However, the administration of ethanol did significantly influence the number of closed arm entries suggesting a change in locomotor activity. When compared to the saline control group, none of the selected doses of ethanol used in the present study significantly influenced the locomotor activity of the animals.

Repeated measures ANOVA showed that the administration of ethanol significantly influenced the number of closed arm entries ($F(4, 27)=3.474$; $p<0.05$; *figure 4.2.*). However, ethanol had no significant effects on the number of open arm entries ($F(4, 27)=1.123$; n.s.; *figure 4.1.*), the percentage of the total arm entries made into the open runways ($F(4, 27) = 0.779$; n.s.; *figure 4.1.*) or the percentage of the total time spent in the open arms ($F(4, 27) = 0.963$; n.s.; *figure 4.2.*). However, *post hoc* analysis of the main effect of ethanol administration on the number of entries into the enclosed arms showed that

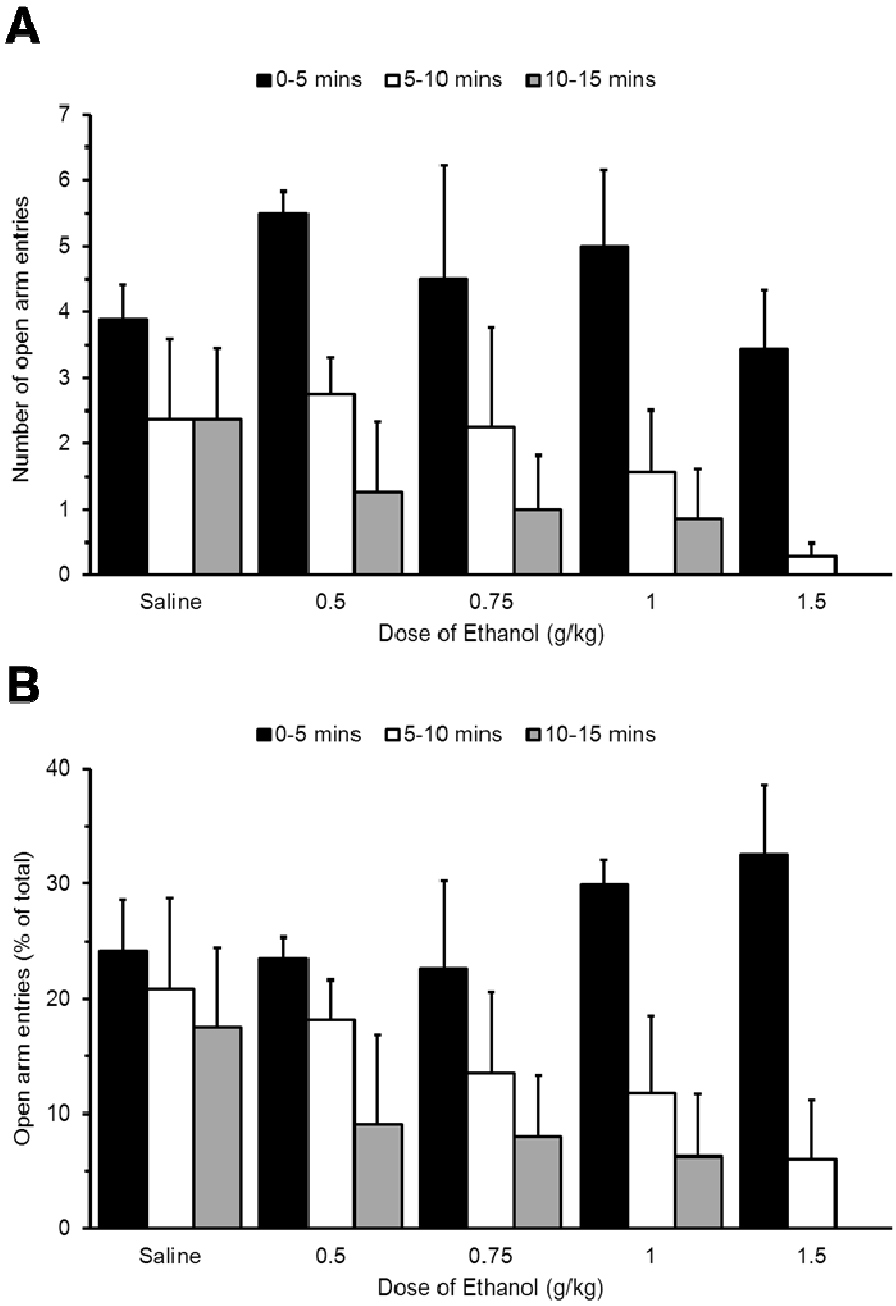

Figure 4.1. – The effect of systemic administration of ethanol on the number and percentage of entries made into the open arms of the elevated plus-maze. Animals were habituated to the injection procedure by administering saline (1 ml/kg daily) for 8 consecutive days in the testing room. On the test day, animals were administered the saline vehicle (*n=8*), 0.5 g/kg (*n=4*), 0.75 g/kg (*n=4*), 1 g/kg (*n=8*) or 1.5 g/kg (*n=8*) ethanol i.p. 20 min prior to testing (6 ml/kg). Behaviour on the elevated plus-maze was scored for 15 min (3 x 5 min time bins). **Panel A** shows the number of entries made into the open runways of the elevated plus-maze; **Panel B** expresses these as a percentage of the total number of arm entries. Data are presented as mean ± S.E.M.

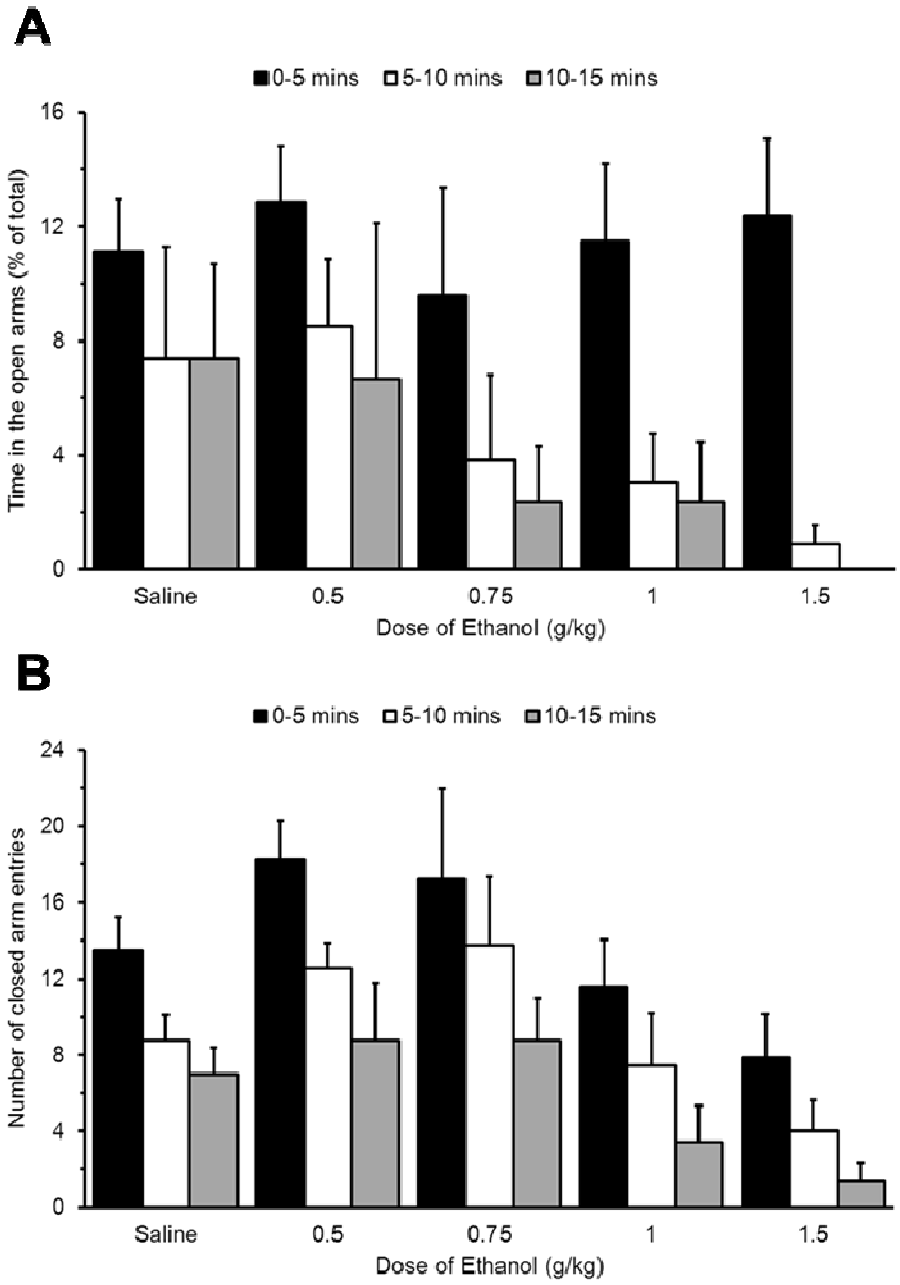

Figure 4.2. – The effect of systemic administration of ethanol on the percentage of the total time spent in the open runways and the number of entries made into the enclosed runways of the elevated plus-maze. Animals were habituated to the injection procedure by administering saline (1 ml/kg daily) for 8 consecutive days in the testing room. On the test day, animals were administered the saline vehicle (*n=8*), 0.5 g/kg (*n=4*), 0.75 g/kg (*n=4*), 1 g/kg (*n=8*) or 1.5 g/kg (*n=8*) ethanol i.p. 20 min prior to testing (6 ml/kg). Behaviour on the elevated plus-maze was scored for 15 min (3 x 5 min time bins). The percentage of the total time spent in the open runways of the elevated plus-maze is shown in **panel A**. The number of entries made into the enclosed runways is shown in **panel B** and was significantly influenced by the administration of ethanol. However, none of the doses of ethanol significantly differed from the saline control and therefore the effects of ethanol on the number of enclosed arm entries are bi-phasic in nature. Data are presented as mean ± S.E.M.

none of the doses of ethanol administered in the present study were significantly different from the saline control.

The number of open arm entries (*Greenhouse-Geisser correction applied*; $F(1.499, 40.485)=30.544$; $p<0.001$), the percentage of total arm entries made into the open runways ($F(2, 54) = 14.672$; $p<0.001$), the percentage of the total time spent in the open runways ($F(2, 54) = 21.243$; $p<0.001$) and the number of entries into the enclosed runways (*Greenhouse-Geisser correction applied*; $F(1.385, 37.384)=72.930$; $p<0.001$) were significantly different over the course of the time bins.

There was no significant interaction the administration of ethanol and the time bins on the number of open arm entries (*time x treatment*; *Greenhouse-Geisser correction applied*; $F(5.998, 40.485)=1.539$; n.s.; *figure 4.1.*), the percentage of entries made into the open runways (*time x treatment*; $F(8, 54) = 1.231$; n.s.; *figure 4.1.*), the percentage of the total time spent exploring the open arms (*time x treatment*; $F(8, 54) = 1.113$; n.s.; *figure 4.2.*) or the number of entries into the enclosed runways (*time x treatment*; *Greenhouse-Geisser correction applied*; $F(5.538, 37.384)=0.536$; n.s.; *figure 4.2.*).

4.1.4. Discussion

Previous studies have reported anxiolytic-like effects in the elevated plus-maze in rats following the intra-peritoneal administration of ethanol (Criswell *et al*, 1994; Moy *et al*, 1997). Similarly, Prunell *et al*. (1994a, 1994b) reported anxiolytic-like effects in the elevated plus-maze in response to the administration of ethanol *per os* in rats. However, the investigation of the anxiolytic properties of ethanol in rats is reported to be limited by the sedative and ataxic effects of ethanol at higher doses (Eckardt *et al*, 1998). In

agreement, the data of the present study do not suggest the significant anxiolytic-like effects previously reported in Sprague-Dawley rats systemically administered ethanol (Criswell *et al*, 1994; Moy *et al*, 1997; Prunell *et al*, 1994a; Prunell *et al*, 1994b). However, the bi-phasic effects of ethanol on the number of enclosed arm entries suggest that, in part, the sedative properties of ethanol may mask the anxiolytic-like effects in the study. In agreement, File (1980) reported the administration 1.2 g/kg ethanol produces sedatory effects in the social interaction test. Similarly, Criswell *et al*. (1994) reported sedatory effects were evident at 1 g/kg in the elevated plus-maze.

Linakis and Cunningham (1979) reported that the administration of ethanol into the peritoneum acts upon the mucosal membrane to cause a dose-dependent irritant effect. In addition, the rate of absorption of the drug follows a bi-phasic trend with increasing concentrations of ethanol via this route of administration (Linakis and Cunningham, 1979). Criswell *et al*. (1994) administered a fixed concentration of ethanol, varying the volume administered by the weight of the animal (ml/kg) to achieve the desired dose in an attempt to account for these potentially confounding factors. Such an approach could have been adopted in the present study. However, additional saline groups would be incorporated into the study to control for the varying volumes of solution administered. Alternatively, LASA guidelines suggest that rats may be administered up to 10 ml/kg via the i.p. administration route (http://www.dilab.com/pdf/lasa_administration.pdf; accessed 27/9/13). Therefore, the concentration of the ethanol administered in the present study could have been further diluted to minimise the aversive component of the administration.

4.2. The effect of intracerebroventricular administration of ethanol on the exploration of the open and closed arms of the elevated plus-maze.

4.2.1. Rationale

Correa *et al*. (2003a) previously reported that the i.p. administration of ethanol in rats suppresses locomotor activity at high doses (2 g/kg). Conversely, the ICV administration (32 µg - 128 µg) of ethanol increased locomotor activity in both the open field and motor activity cages (Correa *et al*, 2003a; 2003b). In addition, the ICV administration of 128 µg of ethanol increased locomotor activity in the central zone of the open field which may reflect an anxiolytic-like effect (Correa *et al*, 2003b). In addition, Correa *et al*. (2003b) identified that the central administration of the two major metabolites of ethanol, namely acetaldehyde and acetate, influenced both anxiety-like behaviours and locomotor activity in the open field. Therefore, the bi-phasic effect of administering ethanol on locomotor activity and the lack of anxiolytic-like effect reported previously in the elevated plus-maze in response to the systemic administration of ethanol (*see section 4.1.*) may reflect the cumulative effects of acetaldehyde, acetate and ethanol. Therefore, the effects of centrally administered ethanol in the elevated plus-maze were investigated in the present study to attempt to dissociate the effects of ethanol in the elevated plus-maze specifically.

4.2.2. Method

Male Sprague-Dawley rats weighing 250-300 g at the beginning of the experiment were implanted with guide cannulae targeted at the lateral ventricle (*see section 2.2 and 2.3*). The rats were left for 10 days in the home cage to recover from the surgery. On day 11 post-surgery, animals were transported to

the testing room. Under gentle retraint, the stylet was replaced with a 30 ga. needle protruding 1.5 mm beyond the tip of the cannula into the lateral ventricle. On day 11 post-surgery, animals were administered 128.8 µg ethanol (n=4) or the aCSF vehicle (1 µl; n=4) over 2 min by means of the needle inserted through the guide cannula. The needle was left in place for a further 2 min to allow for diffusion of the solution from the tip of the injector. Animals were placed in the centre of the elevated plus-maze facing an open runway (*see section 2.7*). Behaviour was recorded for 15 mins on a digital camera and scored as 3 x 5 min time bins (number of open arm entries, percentage of total entries made into the open runways, percentage of total time spent in the open runways and the number of entries into the closed arms; *figure 4.3.*). Upon completion of the behavioural study the rats were killed humanly by cervical dislocation and the injection site was verified histologically in fixed sections of the lateral ventricle by an observer blind to the treatment the rats had received (*figure 4.3.*). The experiment was performed in a single batch of 12 animals. Animals were tested over 3 days, with 4 animals tested in the elevated plus-maze on each day. Of the 12 animals which began the experiment, 2 animals could not be administered the experimental solution due to the blocking of the guide cannulae. An additional 2 animals were removed from the study as the histological investigation identified the administrations were located outwith the lateral ventricle. Data were analysed by repeated measures ANOVA with the administration of ethanol as the between-subjects factor and time as the within-subjects factor. Independent samples t-tests are reported for the investigation of the interactions between the within and between subject factors.

4.2.3. Results

The administration of ethanol into the lateral ventricle significantly increased the percentage of the trial time spent in the open runways of the elevated plus-maze. However, the administration of ethanol did not have a significant influence on either the number or percentage of entries made into the open runways. Previously, the administration of strychnine into the DRN reversed the platform-induced increase in the duration of time spent in the open runways of the elevated plus-maze. Therefore, the ethanol induced increase in the duration of the trial time spent in the open runways may be mediated, in part, by the potentiation of the glycine receptor-mediated inhibition of the serotonergic neurons of the DRN. Furthermore, the administration of ethanol into the lateral ventricle did not increase the number of enclosed arm entries made in the elevated plus-maze. Therefore, this suggests the effect of ethanol on the duration of the trial time spent in the open runways was independent of an overt change in the locomotor activity of the animals.

Repeated measures ANOVA showed that the administration of ethanol significantly increased the percentage of the total time spent in the open runways ($F(1,6) = 8.178$; $p<0.05$; *figure 4.5.*). There was no significant effect of ethanol on the number of open arm entries ($F(1, 6)=5.672$; n.s.; *figure 4.4.*), the percentage of total entries made into the open runways ($F(1,6) = 3.976$; n.s.; *figure 4.4.*) and the number of closed arm entries ($F(1,6) = 0.038$; n.s.; *figure 4.5.*).

The percentage of total entries made into the open runways ($F(2,12) = 7.167$; $p<0.05$), the percentage of the total time spent exploring the open runways ($F(2,12) = 5.001$; $p<0.05$) and the number of enclosed arm entries ($F(2,12) = 22.544$; $p<0.001$) were significantly different between the time bins. However,

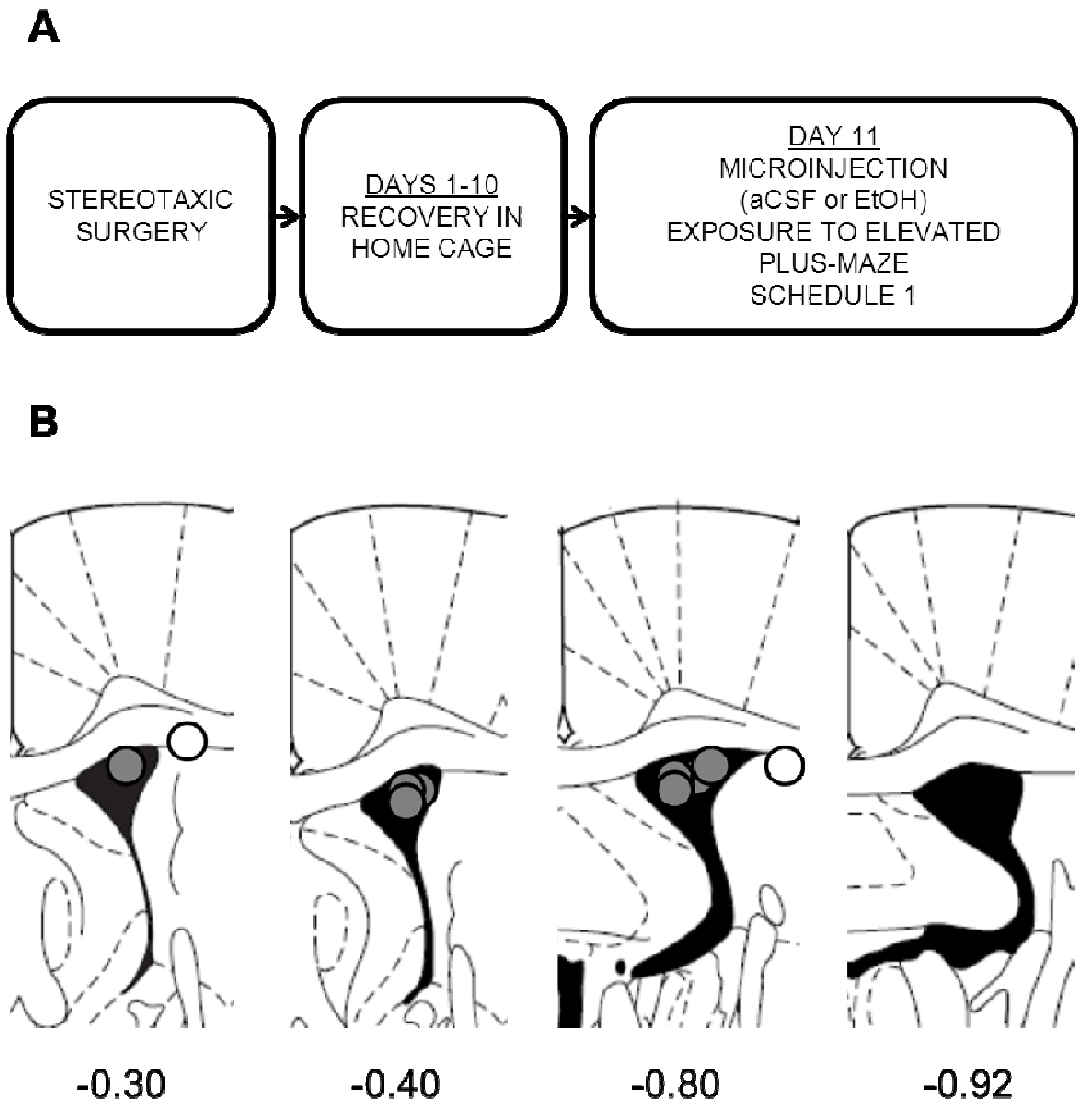

Figure 4.3. – Experimental design and a representative schematic showing the distribution of microinjection sites targeted at the lateral ventricle. Panel A shows the experimental design, where the number of days denotes the number of days post-surgery. **Panel B** is a diagramatic representation of the lateral ventricle and surrounding area adapted from the coronal sections of Paxinos and Watson (1998; **bottom panel**). Numerical values indicate the posterior distance from Bregma. Following behavioural testing and subsequent schedule 1 procedure, 2% Chicago sky blue dye was administered into the lateral ventricle using the same injector used for administration of the testing solution. The brain was removed and fixed in 4% formalin for 48 hr and then transferred to a 30% sucrose solution for 72 hr. Coronal sections (36 µm) containing the lateral ventricle were mounted on gelatinised slides and stained using cresyl violet to visualise the topography of the brain. Filled circles show examples of cannulations which resulted in the successful administration of substances into the lateral ventricle and were included in the study. Open circles depict the location of injection sites excluded from the study due to the administration of substances outside of the lateral ventricle. Histological assessment of the cannulations was performed by an observer blind to the treatment and behavioural history of the animals.

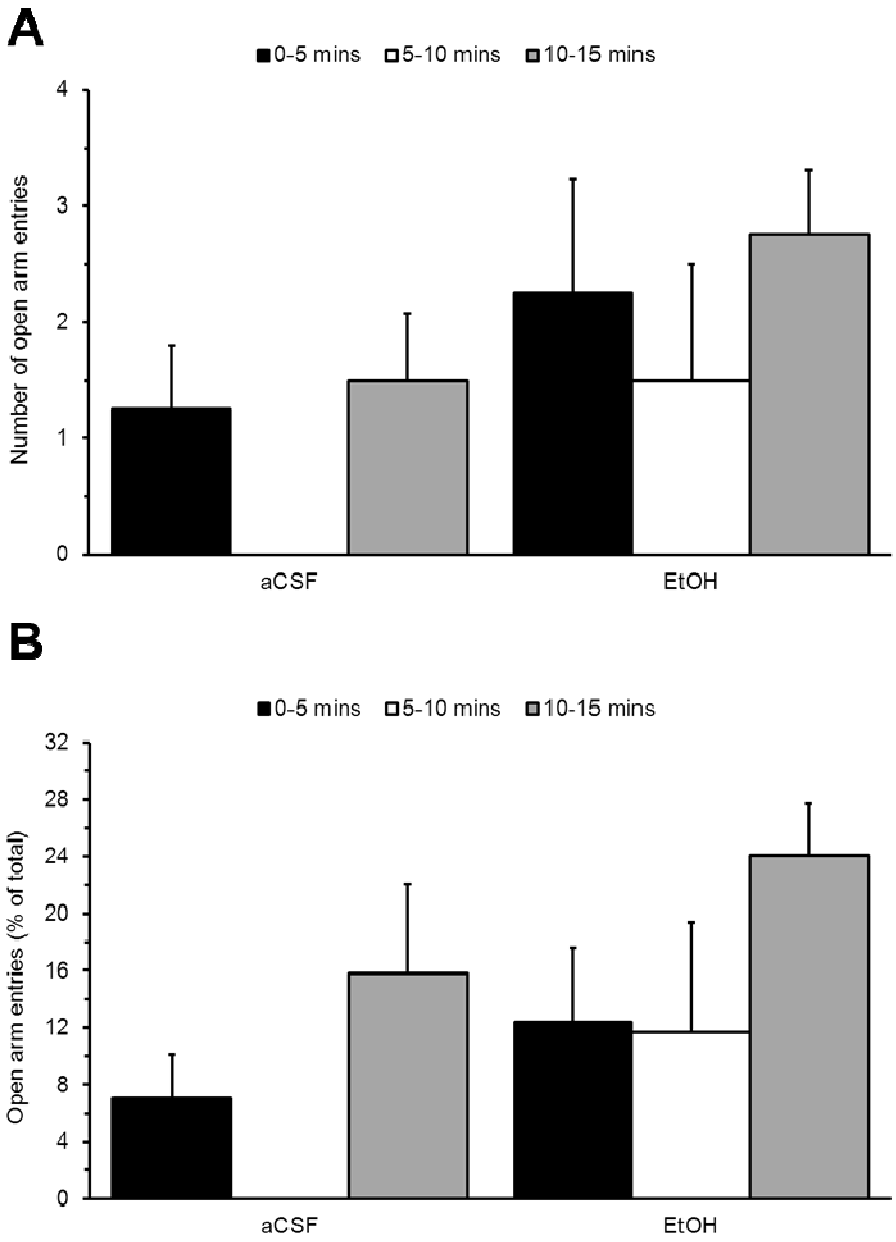

Figure 4.4. – The effect of intracerebroventricular administration of ethanol on the number and percentage of the total entries made into the open runways of the elevated plus-maze. Following surgery, animals remained in the home cage for 10 days to recover. On the 11th day following surgery, animals were administered 128.8 µg ethanol (*n=4*) or the aCSF vehicle (1 µl; *n=4*) into the lateral ventricle via indwelling cannulae prior to a 15 min exposure to the open field. **Panel A** shows the total number of entries made into the open runways of the elevated plus-maze; **Panel B** expresses these open arm entries as a percentage of the total number of entries made into the open and closed arms of the elevated plus-maze. Data are presented as the mean ± S.E.M.

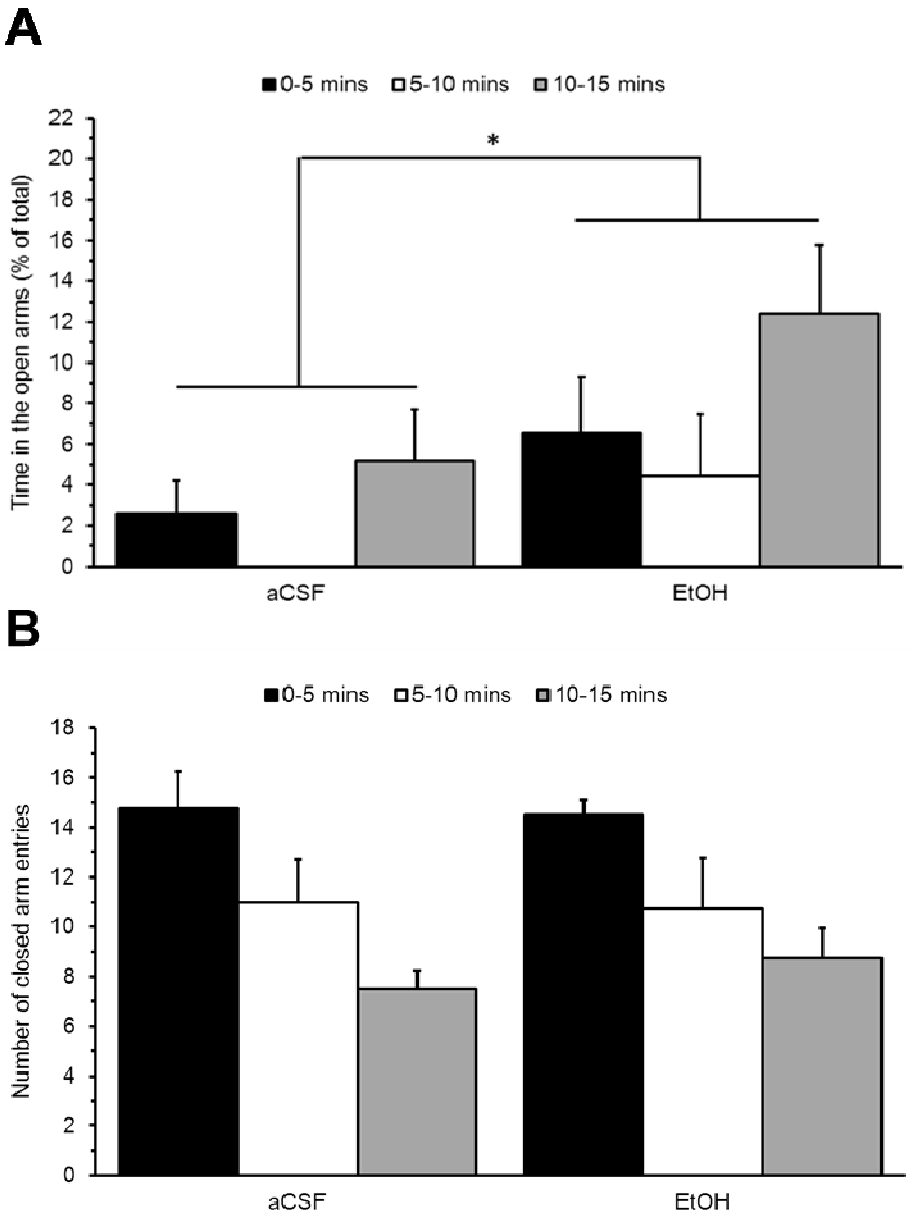

Figure 4.5. – The effect of intracerebroventricular administration of ethanol on the percentage of time spent exploring the open runways and the number of closed arm entries in the elevated plus-maze. Following surgery, animals remained in the home cage for 10 days to recover. On the 11th day following surgery, animals were administered 128.8 µg ethanol (*n=4*) or the aCSF vehicle (1 µl; *n=4*) into the lateral ventricle via indwelling cannulae prior to a 15 min exposure to the open field. **Panel A** shows the percentage of the total time spent exploring the open runways of the elevated plus-maze was increased by the ICV administration of ethanol. **Panel B** expresses the number of entries made into the enclosed runways. $p<0.05$, * versus aCSF control. Data are presented as the mean ± S.E.M.

the number of open arm entries ($F(2, 12)=2.969$; n.s.) was not significantly different between the time bins.

There was no significant influence of an interaction between the administration of ethanol and the time bins on the number of open arm entries (*time x treatment*; $F(2, 12)=0.092$; n.s.; *figure 4.4.*), the percentage of the total arm entries made into the open arms (*time x treatment*; $F(2,12) = 0.34$; n.s.; *figure 4.4.*), the percentage of the total time spent in the open runways (*time x treatment*; $F(2,12) = 0.352$; n.s.; *figure 4.5.*) and the number of closed arm entries (*time x treatment*; $F(2,12) = 0.397$; n.s.; *figure 4.5.*).

4.2.4. Discussion

The data in the elevated plus-maze suggest that the administration of ethanol into the lateral ventricle induced a significant increase in the time spent in the open runways, suggesting an anxiolytic-like effect. In addition, both the number and percentage of the entries made into the open runways approached significance. A power analysis of the data generated in the study suggested that group sizes of 5 animals would be required to avoid a type II statistical error. If these trends were to achieve significance, the ICV administration of ethanol would mimic the anxiolytic-like effects previously reported in the elevated plus-maze in response to systemic administration (Criswell *et al*, 1994; Prunell *et al*, 1994a; Prunell *et al*, 1994b). Contrary to the findings of Correa et al. (2003a; 2003b), the pilot study seems to suggest that the administration of ethanol into the lateral ventricle did not significantly influence the locomotor activity of the animals. However, the absence of an overt change in locomotor activity suggests that the ICV administration of ethanol may serve as an alternative route of administration to study the anxiolytic properties of ethanol in the

absence of the confounding sedatory properties that have previously been reported in rats (Eckardt *et al*, 1998).

4.3. The effect of intra-DRN administration of strychnine and intracerebroventricular administration of ethanol on the exploration of the elevated plus-maze and locomotor activity box.

4.3.1. Rationale

Previous data suggest that the ICV administration of ethanol elicits an anxiolytic-like effect in the elevated plus-maze (*see section 4.2*). Previous studies in the laboratory have identified that the strychnine-sensitive glycine receptors in the DRN are potentiated by low, physiological concentrations of ethanol (30 mM; Maguire *et al*, 2013). Classically, the suppression of the serotonergic neurons of the DRN has been identified as a component of the anxiolytic properties of the benzodiazepines (Pratt *et al*, 1979; Laurent *et al*, 1983; Gonzalez and File, 1997). Therefore, it was hypothesised that a component of the anxiolytic properties of ethanol in the elevated plus-maze may be mediated by the potentiation of the strychnine-sensitive glycine receptors. The hypothesis was investigated by administering strychnine and ethanol into the DRN and lateral ventricle respectively. Behaviour was accessed in the elevated plus-maze and locomotor activity box. The investigation of behaviours in the locomotor activity box was incorporated into the study to investigate the effects of the intra-DRN administration in animals exposed to less aversive environment when compared to the open field and elevated plus-maze tests of anxiety.

4.3.2. Method

Male Sprague-Dawley rats weighing 250-300 g at the beginning of the experiment were implanted with guide cannulae targeted at the lateral ventricle and DRN (*see section 2.2 and 2.3*). The animals were left for 10 days in the home cage to recover. On day 11 post-surgery, animals were transported to the testing room. Under gentle restraint, a stylet was replaced with a 36 ga. needle protruding 1.5 mm beyond the tip of the cannula into the lateral ventricle. Animals were administered 128.8 µg ethanol (n=15) or the aCSF vehicle (1 µl; n=14) into the lateral ventricle by means of the needle inserted via the guide cannula over 4 min. 2 min into the administration of the solutions into the lateral ventricle, a second needle was introduced into the DRN protruding 2 mm from the tip of the guide cannula. Animals were administered 1 µg strychnine hydrochloride (n=15) or the aCSF vehicle (500 nl; n=14) over the remaining 2 min by means of the needle inserted via the guide cannula. Both needles remained in place for a further 2 min to allow for diffusion of the solutions from the tips of the two needles. The needles were then removed and replaced by the stylets. Animals were placed at the centre of an elevated plus maze facing an open arm (*see section 2.7*). Behaviour was recorded on a digital camera for 20 min and scored as 4 x 5 min time bins (number of open arm entries, percentage of total entries made into the open runways, percentage of total time spent in the open runways and the number of entries into the closed arms). Behaviour in the elevated plus-maze was investigated over a 20 min period as opposed to the 15 min trial used previously. Data investigating the effects of the i.c.v. administration of ethanol (*figure 4.4. and figure 4.5*) demonstrated a putative increasing trend in all three anxiety measures over the course of the three time bins which may be a putative anxiolytic-like effect of ethanol. Therefore, the trial time was increased by an additional 5 min to investigate this

putative trend and determine the effects of administering ethanol into the lateral ventricle. The animals were returned to the home cage for 3 days. On day 15 post-surgery, the animals were administered the same combination of treatments as described above (ethanol or aCSF into the lateral ventricle; strychnine or aCSF into the DRN). Immediately after the microinjection, the animals were placed in the centre of a locomotor activity box (*see section 2.6*). The behaviour was recorded for 20 mins and scored as 4 x 5 min time bins (percentage time in the central zone and the number of ambulatory counts). The locomotor activity box was a 45 cm squared box comprising two rows of photobeams located on adjacent walls (*figure 2.3.*). The central zone was defined as the innermost 11.5 photobeams on adjacent walls (29.21 cm square) and the ambulatory counts were registered as the breaking of the photobeams positioned parallel to the floor of the apparatus. On day 17 post-surgery, animals were administered a lethal dose of Euthatal (sodium pentobarbital; i.p.).Upon sedation, the animals were administered 2% Chicago sky blue dye (500 nl) over 2 mins using the needles used to administered the solutions on the testing days. Upon anaesthesia, the animals were perfused transcardially with 4% formalin. The injection site was verified histologically in fixed sections of the lateral ventricle and DRN by an observer blind to the treatment the rats had received (figure 4.6.). The experiment was performed in four batches, each consisting of 16 animals. Animals were tested over 16 days, with 4 animals tested in the elevated plus-maze on each day. Of the 64 animals which began the experiment, 1 animal did not recover upon the termination of the anaesthesia and a further animal damaged the cement cap post-surgery. In addition, 5 animals could not be administered the experimental solution due to the blocking of the guide cannula and a further animals was removed as it fell

from an open runway of the plus-maze. An additional 22 animals were removed from the study as the histological investigation identified the administrations were located outwith the lateral ventricle (5 outwith the lateral ventricle alone, 6 outwith the DRN alone and 11 outwith both the DRN and lateral ventricle). Statistical analysis of the data identified 4 animals which were identified as outliers in the final analysis (i.e. greater than double the standard deviation from the mean). Behaviour was analysed by a repeated measures ANOVA using treatment administered into the lateral ventricle (ethanol or aCSF) and treatment administered into the DRN (strychnine or aCSF) as the between-subjects factors analysed and time as the within-subjects factor. *Post-hoc* independent samples t-tests are reported for the investigation of interactions between the within and between subject factors. Greenhouse-Geisser correction factor applied to data which did not adhere to Mauchly's test of sphericity.

4.3.3. Results

As was identified in previous experiments, the i.c.v. administration of ethanol increased the percentage of the trial time spent in the open runways of the elevated plus-maze suggesting an anxiolytic-like effect. In addition, the administration of ethanol increased the number of entries into the open runways of the elevated plus-maze. However, ethanol did not elicit a similar anxiolytic-like effect in the locomotor activity box as measured by the percentage of the trial time spent exploring the central zone. Conversely, the administration of strychnine into the DRN did not significantly influence the anxiety-like behaviours in the elevated plus-maze. However, the administration of strychnine selectively reduced the number of entries into the enclosed runways of the elevated plus-maze and the number of ambulatory counts in the

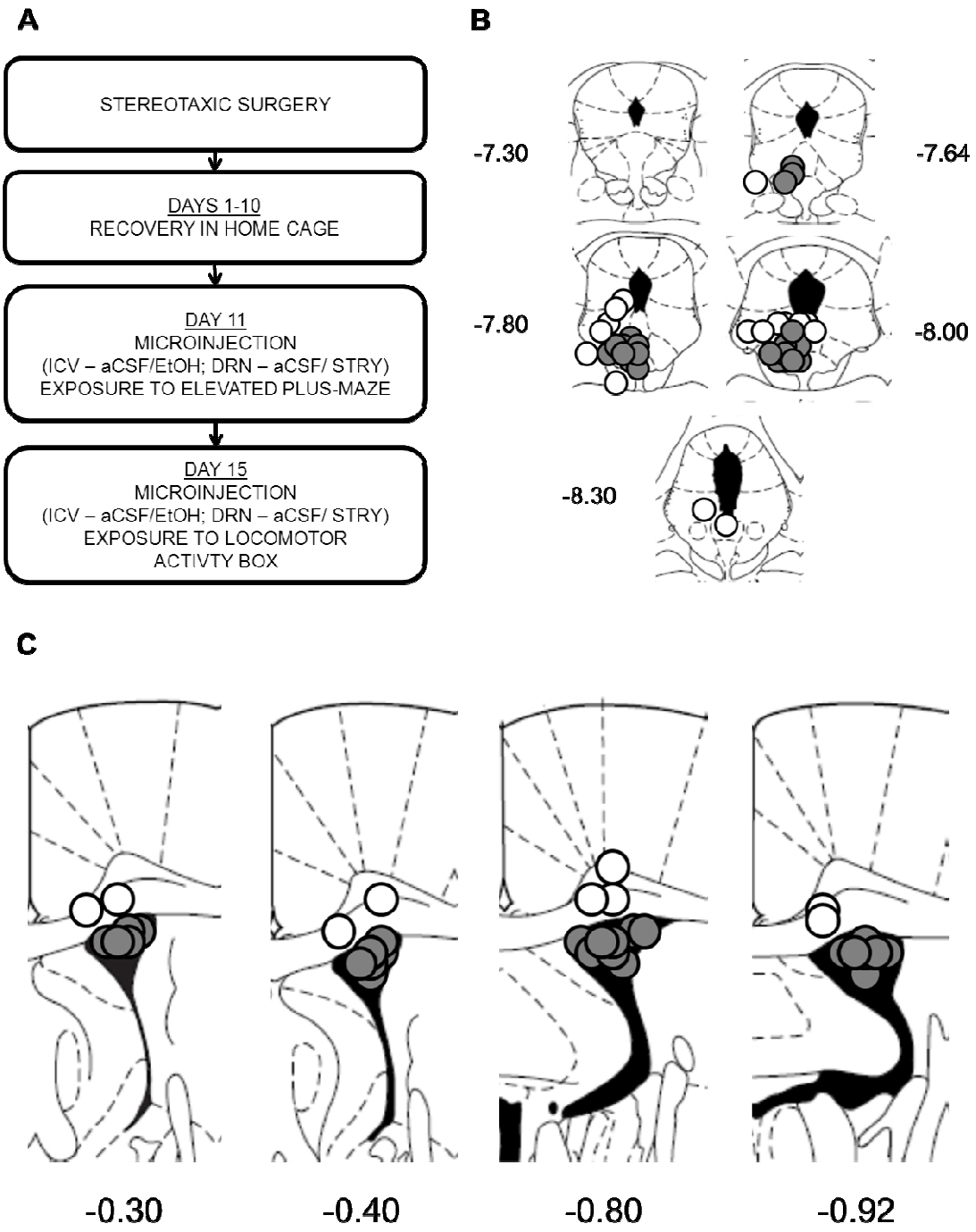

Figure 4.6. – Representative schematic showing the distribution of microinjection sites targeted at the DRN and lateral ventricle. Panel A shows the experimental design, where the number of days denotes the number of days post-surgery. Diagramatic representation of the DRN (**panel B**), the lateral ventricle (**panel C**) and surrounding areas adapted from the coronal sections of Paxinos and Watson (1998). Numerical values indicate the posterior distance from Bregma. Following behavioural testing, 2% Chicago sky blue dye was administered into the DRN and lateral ventricle using the same needles used for administration of the testing solutions. The animals were administered a lethal injection of Euthatal (sodium pentobarbital, 200mg/ml) and were fixed by transcardial perfusion of 4% formalin. The brain was removed and remained in fixative for 48 hr and then transferred to a 30% sucrose solution for 72 hr. Coronal

sections (36 µm) containing the DRN and lateral ventricle were mounted on gelatinised slides and stained using cresyl violet to visualise the topography of the brain. Filled circles show examples of cannulations which resulted in the successful administration of substances into the DRN and lateral ventricle and were included in the study. Open circles depict the location of injection sites excluded from the study either due to the administration of substances outside of the DRN and lateral ventricle. Histological assessment of the cannulations was performed by an observer blind to the treatment and behavioural history of the animals.

locomotor activity box in animals administered aCSF into the lateral ventricle suggesting a suppression of locomotor activity. In the locomotor activity, the administration of strychnine into the DRN reduced the percentage of the trial time spent exploring the central zone suggesting a putative anxiogenic-like effect.

In the elevated plus-maze, there was a main effect of the ICV administration of ethanol on the number of open arm entries ($F(1,25)=5.933$; $p<0.05$; *figure 4.7.*), the percentage of the total time spent exploring the open arms ($F(1,25)=6.886$; $p<0.05$; *figure 4.8.*) and the number of closed arm entries ($F(1,25)=5.140$; $p<0.05$; *figure 4.8.*). However, there was no main effect of ethanol on the percentage of the total arm entries made into the open runways of the elevated plus-maze ($F(1,25)=2.194$; n.s.; *figure 4.7.*). In the locomotor activity box, the ICV administration of ethanol did not significantly influence the percentage of the total time spent in the central zone ($F(1,25)=0.033$; n.s.; *figure 4.9.*) or the total number of ambulatory counts ($F(1,25)=0.095$; n.s.; *figure 4.9.*).

The administration of strychnine into the DRN did not significantly influence the number of open arm entries ($F(1,25)=0.212$; n.s.; *figure 4.7.*), the percentage of the total entries made into the open runways ($F(1,25)=0.781$; n.s.; *figure 4.7.*), the percentage of the total time spent in the open runways ($F(1,25)=0.374$; n.s.;

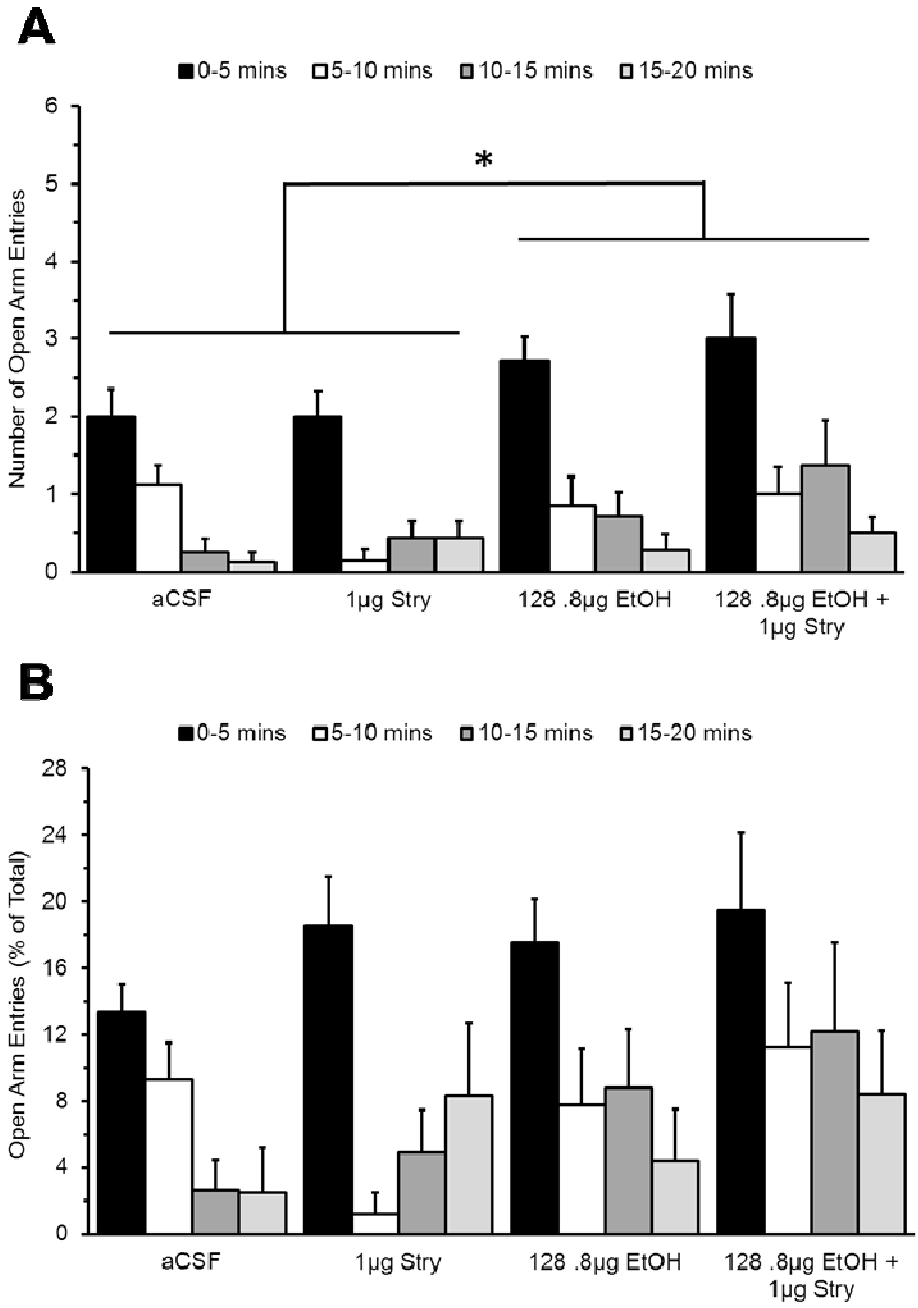

Figure 4.7. – The effect of intracerebroventricular administration of ethanol and intra-DRN administration of strychnine on the number and percentage of the total entries made into the open runways of the elevated plus-maze. On the 11th day post-surgery, animals were administered 128.8 µg ethanol (*n=15*) or the aCSF vehicle (*n=14*; 1 µl) into the lateral ventricle in addition to the administration of 1 µg strychnine hydrochloride (*n=15*) or the aCSF vehicle (*n=14*; 500nl) into the DRN via two indwelling cannulae prior to a 20 min exposure to the elevated plus-maze. **Panel A** shows the number of open arm entries, which was increased by the administration of ethanol ($p<0.05$). **Panel B** expresses the open arm entries as a percentage of the total number of entries. $p<0.05$, * versus aCSF-lateral ventricle control. Data are presented as mean ± S.E.M.

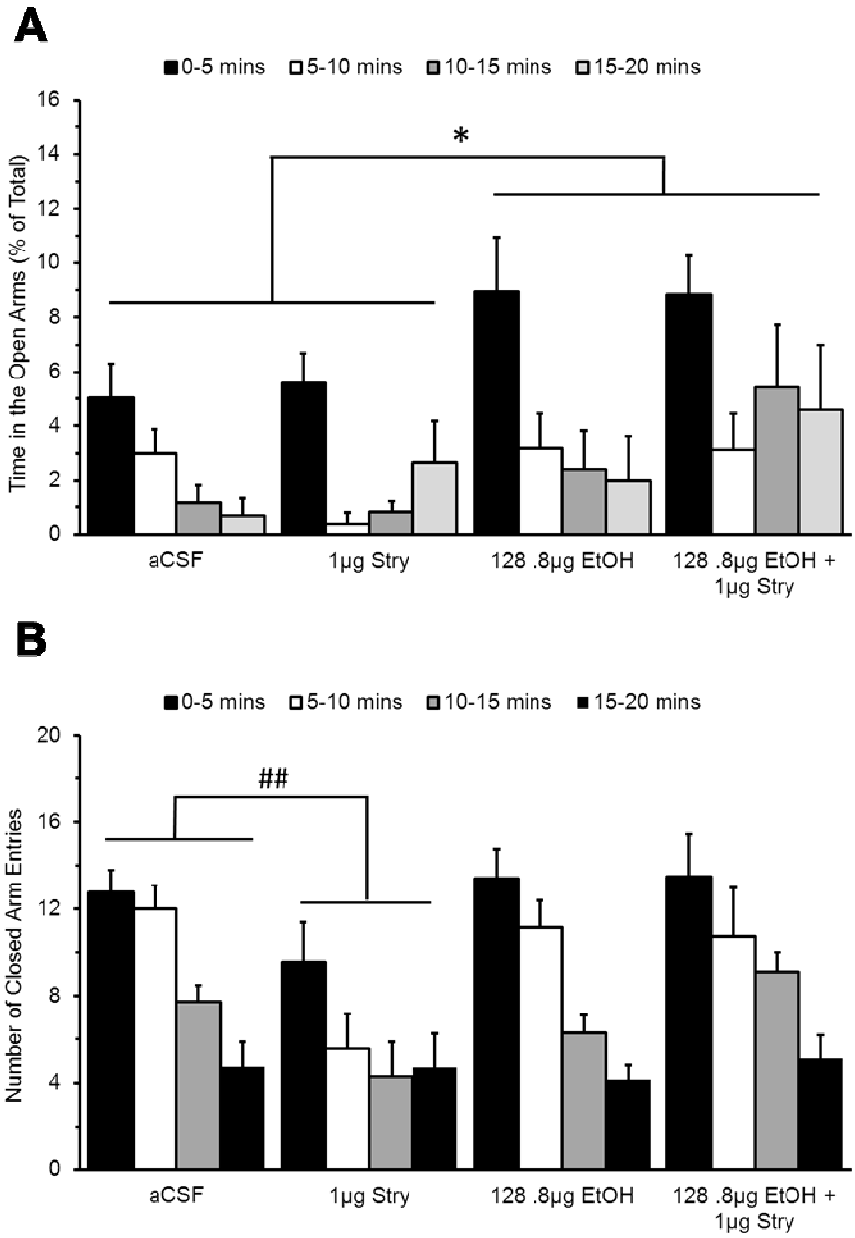

Figure 4.8. – The effect of intracerebroventricular administration of ethanol and intra-DRN administration of strychnine on the percentage of time spent in the open runways and the number of entries into the enclosed arms of the elevated plus-maze. On the 11th day post-surgery, animals were administered 128.8 µg ethanol (*n=15*) or the aCSF vehicle (*n=14*; 1 µl) into the lateral ventricle in addition to the administration of 1 µg strychnine hydrochloride (*n=15*) or the aCSF vehicle (*n=14*; 500nl) into the DRN via two indwelling cannulae prior to a 20 min exposure to the elevated plus-maze. **Panel A** shows the percentage of the total time spent exploring the open runways of the elevated plus-maze, which was increased by the administration of ethanol ($p<0.05$). **Panel B** shows the number of entries into the enclosed runways was significantly reduced by the administration of strychnine in rats that were administered aCSF, but not ethanol, into the lateral ventricle ($p<0.01$). $p<0.05$, * versus aCSF-lateral ventricle control; $p<0.01$, ## versus aCSF-DRN control. Data are presented as mean ± S.E.M.

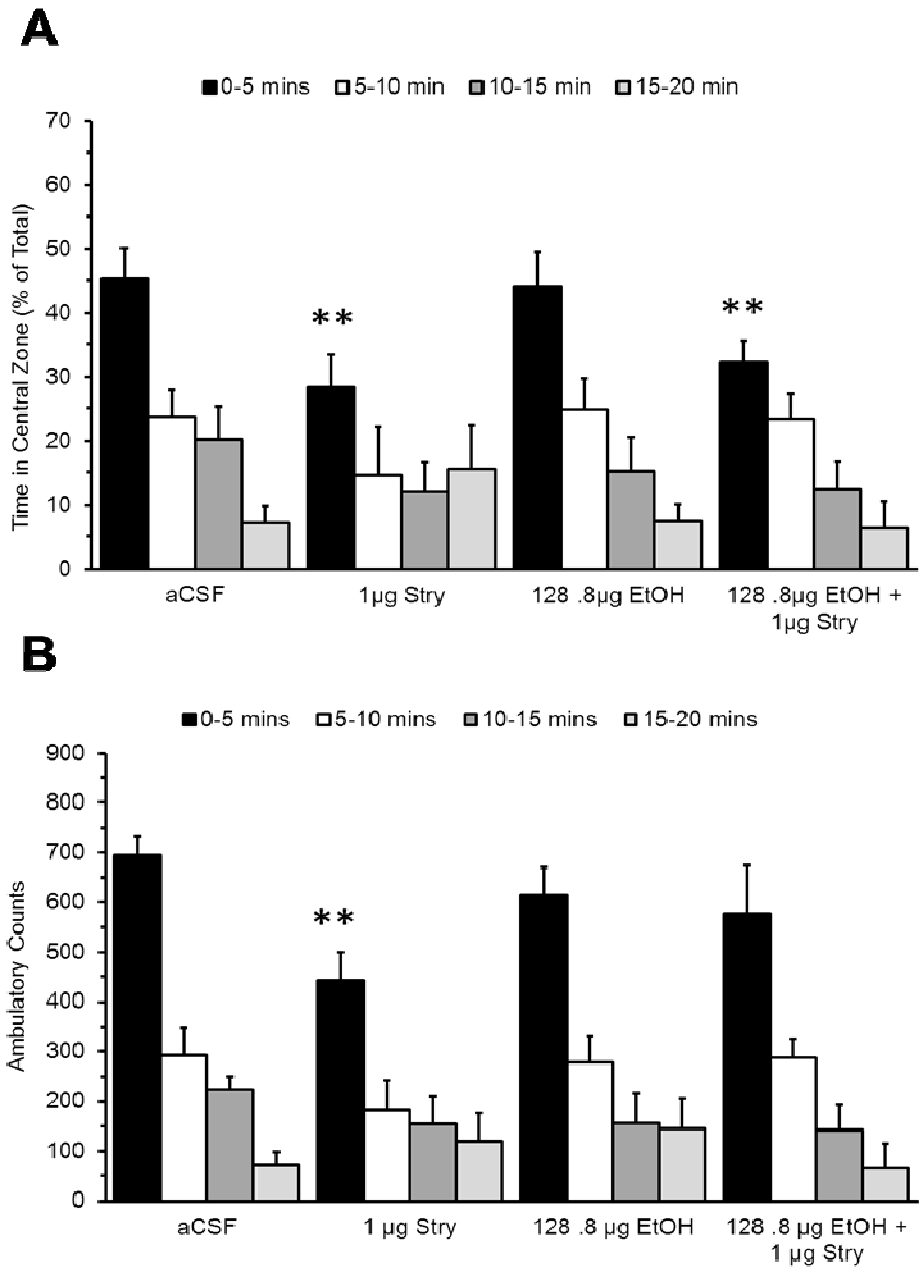

Figure 4.9. – The effect of intracerebroventricular administration of ethanol and intra-DRN administration of strychnine the percentage of time spent exploring the central zone and number of ambulatory counts made in the locomotor activity box. On the 15th day post-surgery, animals were administered 128.8 µg ethanol (*n=15*) or the aCSF vehicle (*n=14*; 1 µl) into the lateral ventricle in addition to the administration of 1 µg strychnine hydrochloride (*n=15*) or the aCSF vehicle (*n=14*; 500nl) into the DRN via two indwelling cannulae prior to a 20 min exposure to locomotor activity box. **Panel A** shows the percentage of the total time spent exploring the central zone of the locomotor activity box, which was significantly reduced in the 0-5 min time bin by the administration of strychnine ($p<0.01$). **Panel B** shows the number of ambulatory counts in the locomotor activity box, which was reduced in the 0-5 min time bin by the administration of strychnine in rats which were administered aCSF, but not ethanol, into the lateral ventricle ($p<0.01$). $p<0.01$, ** versus aCSF-DRN control. Data are presented as mean ± S.E.M.

figure 4.8.) or the number of closed arm entries ($F(1,25)=1.606$; n.s.; *figure 4.8.*) in the elevated plus-maze. Similarly, there were no significant effects of strychnine on the percentage of the total time spent in the central zone ($F(1,25)=1.876$; n.s.; *figure 4.9.*) or on the total ambulatory counts ($F(1,25)=2.028$; n.s.; *figure 4.9.*) in the locomotor activity box.

There was no significant interaction between the administration of ethanol and the administration of strychnine on the number of open arm entries (*ethanol x strychnine*; $F(1,32)=1.389$; n.s.; *figure 4.7.*), the percentage of the total arm entries made into the open runways (*ethanol x strychnine*; $F(1,25)=0.245$; n.s.; *figure 4.7.*) and the percentage of the total time spent in the open arms (*ethanol x strychnine*; $F(1,25)=0.821$; n.s.; *figure 4.8.*) of the elevated plus-maze. However, there was a significant interaction between the administration of ethanol and the administration of strychnine on the number of closed arm entries (*ethanol x strychnine*; $F(1,25)=5.792$; $p<0.05$; *figure 4.8.*) in the elevated plus-maze. In the locomotor activity box, there was no significant interaction between the administration of ethanol and the administration of strychnine on the percentage of the total time spent in the central zone (*ethanol x strychnine*; $F(1,25)=0.015$; n.s.; *figure 4.9.*) or the number of ambulatory counts (*ethanol x strychnine*; $F(1,25)=0.509$; n.s.; *figure 4.9.*). *Post hoc* analysis of the effect of the interaction between the administration of ethanol and the administration of strychnine on the number of entries into the enclosed arms of the elevated plus-maze found that the administration of strychnine into the DRN significantly reduced the number of closed arm entries in rats administered aCSF ($t(12) = 3.645$, $p<0.01$), but not ethanol ($t(13) = -0.676$, n.s.), into the lateral ventricle.

The number of open arm entries ($F(3,75)=42.767$; $p<0.001$), the percentage of the total arm entries made into the open runways ($F(3,75)=16.537$; $p<0.001$),

the percentage of the total time spent in the open runways ($F(3,75)=13.986$; $p<0.001$) and the number of closed arm entries (*Greenhouse-Geisser correction*; $F(2.148, 53.699) = 30.267$; $p<0.001$) were significantly different between the time bins in the elevated plus-maze. Similarly, the percentage of the total time spent in the central zone ($F(3,75)=51.548$; $p<0.001$) and the total number of ambulatory counts ($F(3,75)=101.073$; $p<0.001$) varied significantly over the time bins.

The number of open arm entries (*time x ethanol*; $F(3, 75)=1.491$; n.s.; *figure 4.7.*), the percentage of the total arm entries made into the open runways (*time x ethanol*; $F(3, 75)=0.893$; n.s.; *figure 4.7.*), the percentage of the total time spent in the open arms (*time x ethanol*; $F(3,75)=0.693$; n.s.; *figure 4.8.*) and the number of closed arm entries (*time x ethanol*; *Greenhouse-Geisser correction*; $F(2.148, 53.699) = 0.884$; n.s.; *figure 4.8.*) were not significantly influenced by an interaction between the administration of ethanol and the time bins in the elevated plus-maze. In the locomotor activity box, there was no significant interaction between the administration of ethanol and the time bins on the percentage of the total time spent in the central zone (*time x ethanol*; $F(3,75)=1.503$; n.s.; *figure 4.9.*) or the number of ambulatory counts (*time x ethanol*; $F(3,75)=0.879$; n.s.; *figure 4.9.*).

Similarly, there was no significant interaction between the administration of strychnine into the DRN and the time bins on the number of open arm entries (*time x strychnine*; $F(3,75)=1.624$; n.s.; *figure 4.7.*), the percentage of the total arm entries made into the open runways (*time x strychnine*; $F(3,75)=1.515$; n.s.; *figure 4.7.*), the percentage of the total time spent exploring the open runways (*time x strychnine*; $F(3,75)=1.613$; n.s.; *figure 4.8.*) and the number of closed arm entries (*time x strychnine*; *Greenhouse-Geisser correction*; $F(2.148,$

53.699) = 1.937; n.s.; *figure 4.8.*). In the locomotor activity box, there was a significant interaction between the administration of strychnine and the time bins on the percentage of the total time spent in the central zone (*time x strychnine*; $F(3,75)=4.777$; $p<0.01$; *figure 4.9.*). However, the number of ambulatory counts was not significantly influenced by an interaction between the administration of strychnine and the time bins (*time x strychnine*; $F(3,75)=1.688$; n.s.; *figure 4.9.*). *Post hoc* analysis showed that strychnine administered into the DRN significantly reduced the time spent in the central zone, when compared to the aCSF control, during the 0-5 min time bin ($t(27)=3.282$; $p<0.01$; *figure 4.9.*). However, there was no significant effect of intra-DRN administration of strychnine on the percentage of time spent in the central zone in the 5-10 min ($t(27)=0.788$; n.s.), 10-15 min ($t(27)=0.948$; n.s.) or 15-20 min time bins ($t(27)=-0.900$; n.s.).

In addition, there were no significant interaction between the administration of ethanol, the administration of strychnine and the time bins on the number of open arm entries (*time x ethanol x strychnine*; $F(3,75)=0.805$; n.s.; *figure 4.7.*), the percentage of the total arm entries made into the open runways (*time x ethanol x strychnine*; $F(3,75)=1.713$; n.s.; *figure 4.7.*), the percentage of the total time spent in the open runways (*time x ethanol x strychnine*; $F(3,75)=0.577$; n.s.; *figure 4.8.*) and the number of closed arm entries (*time x ethanol x strychnine*; *Greenhouse-Geisser correction*; $F(2.148, 53.699) = 0.987$; n.s.; *figure 4.8.*). However, there was a significant interaction between the administration of ethanol, the administration of strychnine and the time bins on the number of ambulatory counts (*time x ethanol x strychnine*; $F(3,75)=2.770$; $p<0.05$; *figure 4.9.*) but not the percentage of the total time spent in the central zone of the locomotor activity box (time x ethanol x strychnine; $F(3,75)=1.206$;

n.s.; *figure 4.9.*). *Post hoc* analysis showed that in animals administered the aCSF control into the lateral ventricle, the intra-DRN administration of strychnine significantly reduced the number of ambulatory counts in the initial 0-5 min period ($t(12)=3.578$; $p<0.01$; *figure 4.9.*), but had no significant effect on the 5-10 min ($t(12)=1.090$; n.s.), 10-15 min ($t(12)=0.851$; n.s.) or 15-20 min time bins ($t(12)=-0.871$; n.s.). However, in animals administered ethanol into the lateral ventricle, the intra-DRN administration of strychnine had no significant effects on the number of ambulatory counts in the 0-5 min ($t(13)=0.734$; n.s.), 5-10 min ($t(13)=0.901$; n.s.), 10-15 min ($t(13)=0.814$; n.s.) or 15-20 min time bins ($t(13)=0.300$; n.s.).

4.3.4. Discussion

In agreement with the study and Correa *et al*. (2003b), the ICV administration of ethanol induced an anxiolytic-like effect as characterised in the present study by the increase in the duration of time spent exploring and increase in entries to the open runways of the elevated plus-maze. Surprisingly, the ICV administration of ethanol did not increase the percentage of the total entries made into the open arms in the elevated plus-maze. However, this may have been due to the decrease in the number of closed arm entries, which is a denominator in the calculation of the percentage of total entries made into the open runways, observed in animals administered strychnine into the DRN and aCSF into the lateral ventricle. This contrasts with the findings of the previous study in the elevated plus-maze, which investigated animals naïve and repeatedly exposed to an elevated platform stressor, where the administration of strychnine elicited no overt effects on locomotor activity in either group. This supports the hypothesis that some of the properties of strychnine administered into the DRN were masked in prior experiments by the high basal level of

anxiety in the animals. The mechanism responsible for the unveiling of this locomotor effect can only speculated upon. However, they may be attributed to an improvement in the microinjection procedure, surgical technique or post-operative care.

Interestingly, the strychnine-mediated reduction in locomotor activity observed in the animals administered aCSF into the lateral ventricle was not apparent in animals administered ethanol in the elevated plus-maze. One potential explanation for this may be effects of ethanol at a distal site, such the dopaminergic system. As previously discussed (*section 1.4.3.*), the efferent projections which originate from the DRN influence both the origins and terminal domains of the nigrostriatal and mesolimbic dopaminergic systems (Vertes, 1991; Vertes and Linley, 2007; Stratford and Wirtshafter. 1990). However, due to order in which the substances were administered, the ICV administration of ethanol may elicit effects within the dopaminergic system which the subsequent strychnine-mediated facilitation of serotonergic transmission is incapable of inhibiting. For example, Arizzi-LaFrance *et al*. (2006) have previously reported that the direct administration of 16 – 128 µg of ethanol into the substantia nigra pars reticulate mediates a stimulation of locomotor activity in rats exposed to a locomotor activity box. In addition, the administration of low doses of ethanol has previously been reported to increase the firing rate of mesolimbic dopaminergic neurons (Mereu *et al*, 1984; Gessa *et al*, 1985).

In the locomotor activity box, the administration of ethanol had no effect on the ambulatory counts or the percentage of the trial time spent in the central zone. These findings contradict those of Correa *et al*. (2003a; 2003b), who reported that the ICV administration of ethanol elicits a stimulation of locomotor activity and an increase in the number of line crossings in the central zone of an open

field. This may have been due to the dimensions of the locomotor activity box itself, which was smaller than open field used by Correa *et al*. (2003b; 113 cm squared), as the animals administered aCSF and EtOH in the lateral ventricle (in the absence of strychnine) explore the central zone approximately 45% of the duration of the 0-5 min time bin. Therefore, the apparatus may not be sufficiently aversive to elicit an anxiolytic-like effect.

Conversely, the intra-DRN administration of strychnine reduces the percentage of the trial time spent exploring the central zone in the initial 0-5 min time bin suggesting an anxiogenic-like effect. However, this interpretation must be considered cautiously as the ambulatory counts are also reduced in the initial 0-5 min time in animals administered strychnine into the DRN and aCSF into the lateral ventricle. As was observed in the elevated plus-maze, the administration of ethanol prevented, in part, the decrease in locomotor activity induced by the intra-DRN administration of strychnine in the 0-5 min time bin. However, the ICV administration of ethanol did not reverse the strychnine-mediated decrease in the duration of the 0-5 min time bin spent exploring the central zone. Therefore, it may be that the strychnine-sensitive glycine receptors expressed in the DRN may mediate distinct anxiolytic and stimulatory roles.

The stimulatory and inhibitory effects of serotonin on locomotor activity have previously been discussed (*section 1.3.1.*) and Boureau and Dayan (2011) have suggested these effects may be mediated by interactions with the dopaminergic system. Topographically, the organisation of the serotonergic efferent projections originating from the DRN would support the hypothesis that the strychnine-sensitive glycine receptors may mediate both locomotor and anxiety-like responses (*section 1.4.3.*). While the dorsal and medial ascending pathways predominantly terminate in the striatum, globus pallidus and

substantia nigra (Michelsen *et al*, 2007); the ventral ascending pathway terminates in centres implicated in the mediation of anxiety-like behaviours such as the hypothalamic nuclei, the septum, the amygdala, cortical centres and the hippocampus (Michelsen *et al*, 2007).

The data from the present study do not support the hypothesis of a functional interaction between the strychnine-sensitive glycine receptors and ethanol. However, there are several factors associated with the experimental design which may account for this. As previously discussed above, the order of drug administration may have influenced the behavioural outcomes. Previously, Maguire *et al*. (2013) reported that the ethanol mediated-potentiation of the strychnine-sensitive glycine receptors in the DRN was achieved at a concentration of 30 mM. In agreement, Mascia *et al*, (1996) reported that glycine induced currents in recombinantly expressed glycine receptors were potentiated by ethanol concentrations as low as 10 mM. However, the use of intracerebral microinjection as the route of administration makes the estimation of the concentration range within the DRN difficult to access and may be out with the range in which glycinergic inhibition is potentiated. Furthermore, the ethanol-mediated allosteric modulation of glycine receptor function is, similarly to strychnine, dependent upon the presence of the endogenous agonist.

4.4. The effect of intra-DRN administration of ethanol on the exploration of the open and closed arms of the elevated plus-maze.

4.4.1. Rationale

The ICV administration of ethanol elicited an anxiolytic-like effect when measured in the elevated plus-maze in prior experiments. However, the anxiolytic-like effects of ethanol in the elevated plus-maze, when administered

into the lateral ventricle, did not appear to be sensitive to the antagonism of strychnine. Therefore, the administration of ethanol into the DRN was investigated in the elevated plus-maze. Previously, the administration of ethanol has been reported to suppress the neuronal excitation of the serotonergic neurons of the DRN in anaesthetised animals (Chu *et al*, 1984; Pistis *et al*, 1997b). Therefore, the hypothesis was that the local administration of ethanol would elicit an anxiolytic-like effect.

4.4.2. Method

Male Sprague-Dawley rats weighing 250-300 g at the beginning of the experiment were implanted with guide cannulae targeted at the DRN (*see section 2.2 and 2.3*). The rats were left for 10 days in the home cage to recover. On day 11 post-surgery, animals were transported to the testing room. Under gentle retraint, the stylet was replaced with a 36 ga. needle protruding 2 mm beyond the tip of the cannula into the DRN. On day 11 post-surgery, animals were administered 4 µg (n=5), 8 µg (n=9), 16 µg ethanol (n=7) or the aCSF vehicle (500 nl; n=8) into the DRN over 2 mins by means of the needle inserted via the guide cannula. The needle was left in place for a further 2 min to allow for diffusion of the solution from the tip of the injector. Following the administration of substances, the needle was replaced by the stylet. Animals were placed at the centre of an elevated plus maze facing an open arm (*see section 2.7*). Behaviour was recorded on a digital camera for 15 min and scored as 3 x 5 min time bins (number of open arm entries, percentage of total entries made into the open runways, percentage of total time spent in the open runways and the number of entries into the closed arms). Upon completion of the behavioural study the rats were killed humanly by cervical dislocation and the injection site was verified histologically in fixed sections of the injection site

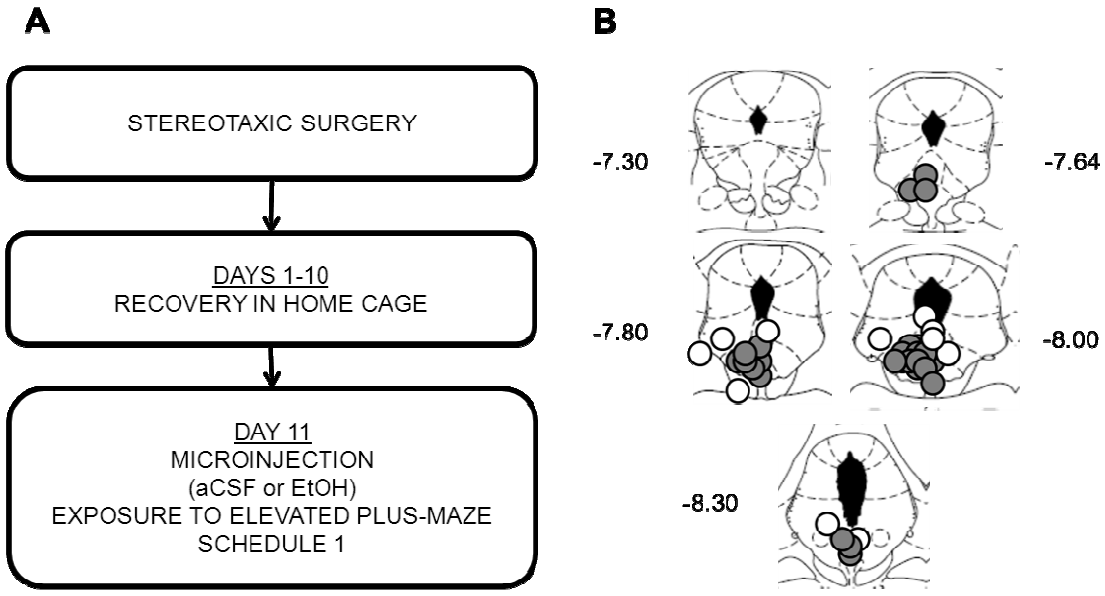

Figure 4.10. – Representative schematic showing the distribution of microinjection sites targeted at the DRN. Panel A shows the experimental design, where the number of days denotes the number of days post-surgery. **Panel B** is a diagramatic representation of the DRN and surrounding area adapted from the coronal sections of Paxinos and Watson (1998). Numerical values indicate the posterior distance from Bregma. Following behavioural testing and subsequent schedule 1 procedure, 2% Chicago sky blue dye was administered into the DRN using the same injector used for administration of the testing solution. The brain was removed and fixed in 4% formalin for 48 hr and then transferred to a 30% sucrose solution for 72 hr. Coronal sections (36 µm) containing the DRN were mounted on gelatinised slides and stained using cresyl violet to visualise the topography of the brain. Filled circles show examples of cannulations which resulted in the successful administration of substances into the DRN and were included in the study. Open circles depict the location of injection sites excluded from the study either due to the administration of substances outside of the DRN or by the occlusion of the cerebral aqueduct. Histological assessment of the cannulations was performed by an observer blind to the treatment and behavioural history of the animals.

was verified histologically in fixed sections of the DRN by an observer blind to the treatment the rats had received (*figure 4.10.*). The experiment was performed in three batches of 16, 24 and 8 animals respectively. Animals were tested over 12 days, with 4 animals tested in the elevated plus-maze on each day. Of the 48 animals which began the study, 2 animals were administered a higher dose of 16 µg ethanol (data not shown), which resulted in an adverse

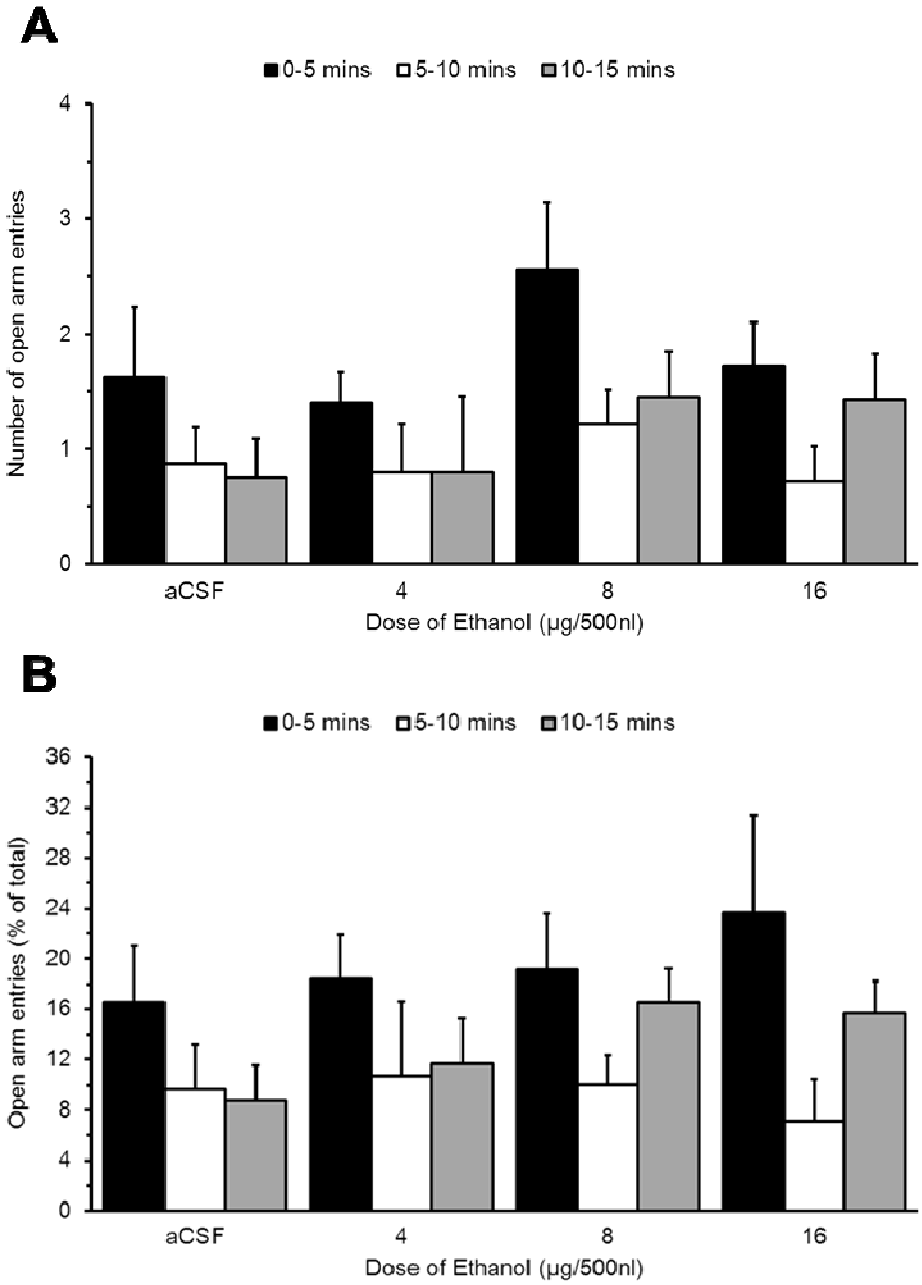

Figure 4.11. – The effect of intra-DRN administration of ethanol on the number and the percentage of entries made into the open arm entries in the elevated plus-maze. Following surgery, animals remained in the home cage for 10 days to recover. On the 11th day following surgery, animals were administered 4 µg (*n=5*), 8 µg (*n=9*) or 16 µg ethanol (*n=7*) or the aCSF vehicle (500 nl; *n=8*) into the DRN via indwelling cannulae prior to a 15 min exposure to the elevated plus-maze. Panel A shows the total number of entries made into the open runways of the elevated plus-maze; panel B expresses these as a percentage of entries made into the open and closed arms of the elevated plus-maze. Data are presented as the mean ± S.E.M.

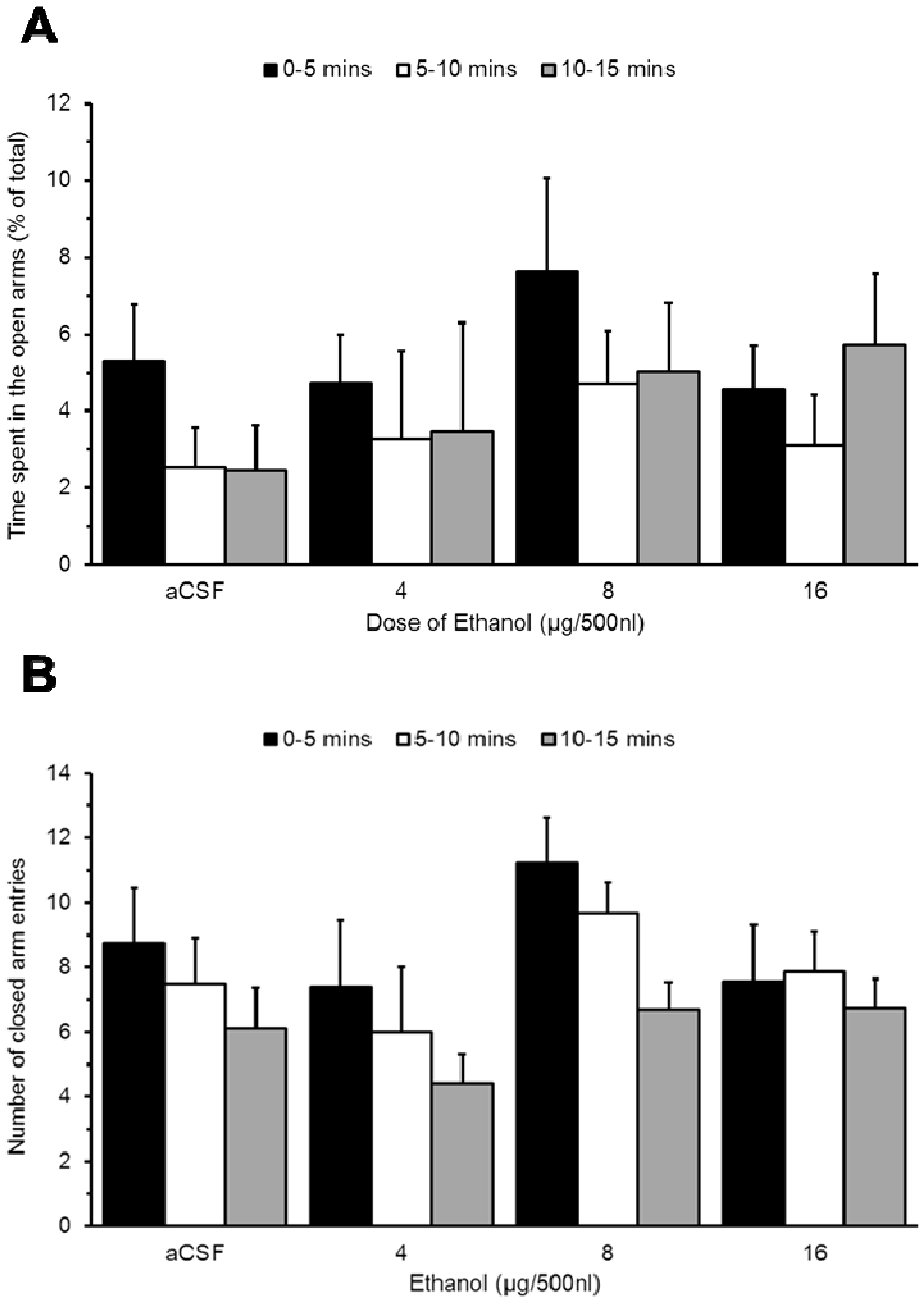

Figure 4.12. – The effect of intra-DRN administration of ethanol on the percentage of the total time spent in the open runways and the number of closed arm entries in the elevated plus-maze. Following surgery, animals remained in the home cage for 10 days to recover. On the 11th day following surgery, animals were administered 4 µg (*n=5*), 8 µg (*n=9*) or 16 µg ethanol (*n=7*) or the aCSF vehicle (500 nl; *n=8*) into the DRN via indwelling cannulae prior to a 15 min exposure to the elevated plus-maze. **Panel A** shows the percentage of the total time spent exploring the open runways of the elevated plus-maze. **Panel B** shows the total number of entries made into the enclosed runways of the elevated plus-maze. Data are presented as the mean ± S.E.M.

response and therefore no further animals were included in the study to investigate this dose of ethanol. In addition, 4 animals could not be administered the experimental solution on the test day as the cannulae became blocked. Histological identification of the target site identified 10 animals where the tip of microinjection needle was located outwith the target site. A further 3 animals were identified as statistical outliers in the final analysis. The behaviour was analysed by a repeated measures ANOVA using treatment (dose of ethanol or aCSF) as the between-subjects factor analysed and time as the within-subjects factor.

4.4.3. Results

Ethanol was administered into the DRN to investigate the effect of potentiating the strychnine-sensitive glycine receptor-mediated inhibition of the serotonergic neurons. Therefore, it was hypothesised that the intra-DRN administration of ethanol would induce an anxiolytic-like response in the elevated plus-maze. However, the local administration of ethanol into the DRN did not significantly influence either the indices of anxiety-like behaviour or locomotor activity in the elevated plus-maze.

Repeated measures ANOVA showed there was no main effect of ethanol on the number of open arm entries ($F(3,25)=1.923$; n.s.; *figure 4.11.*), the percentage of total entries made into the open runways ($F(3,25)=0.437$; n.s.; *figure 4.11.*), the percentage of total time spent exploring the open runways ($F(3,25)=1.261$; n.s.; *figure 4.12.*) and the number of closed arm entries ($F(3,25)=2.154$; n.s.; *figure 4.12.*).

The number of open arm entries ($F(2,50)=5.547$; $p<0.01$), the percentage of total entries made into the open runways ($F(2,50)=6.237$; $p<0.01$) and the

number of closed arm entries ($F(2,50)=5.864$; $p<0.01$) varied significantly over the time bins. However, the percentage of the total time spent exploring the open runways was not significantly different across the time bins ($F(2,50)=1.867$; n.s.).

There was no significant interaction between the administration of ethanol and the time bins on the number of open arm entries (*time x ethanol*; $F(6,50)=0.385$; n.s.; *figure 4.11.*), the percentage of total entries made into the open runways (*time x ethanol*; $F(6,50)=0.494$; n.s.; *figure 4.11.*), the percentage of total time spent exploring the open runways (*time x ethanol*; $F(6,50)=0.379$; n.s.; *figure 4.12.*) and the number of closed arm entries (*time x ethanol*; $F(6,50)=0.528$; n.s.; *figure 4.12.*).

4.4.4. Discussion

The administration of ethanol directly into the DRN did not significantly influence any of the anxiety-like behaviours in the elevated plus-maze or alter the locomotor activity of the animals. Previously, Arizzi-LaFrance *et al*. (2006) directly administered 16 – 128 µg of ethanol into the substantia nigra pars reticulate and reported a stimulation of locomotor activity in rats exposed to a locomotor activity box. Comparable, but modestly lower, doses were administered into the DRN in the present study. However, the lack of measurable behavioural outcomes when ethanol was administered into the DRN may be due to the lipophilic nature of ethanol (Greenshaw, 1986). The local administration of ethanal infused directly into the DRN may diffuse rapidly from the target site. However, the administration of higher doses may induce necrosis at the target site.

5. General Discussion

The primary aim of the present study was to investigate the *in vivo* role of the strychnine-sensitive glycine receptors expressed on the serotonergic neurons of the DRN. Collectively, the data support the hypothesis that the strychnine-sensitive glycine receptors mediate anxiety-like behaviours, however, the effects of strychnine on anxiety-like behaviours in rats appear to be dependant upon the basal anxious state of the animal. In agreement, the administration of strychnine into the DRN of animals exposed to a weakly aversive environment, such as the locomotor activity box, or in animals previously habituated to a homologous stressor (i.e. the elevated open platform stressor) conveyed sensitivity to the anxiogenic-like effects mediated by the administration of strychnine into the DRN. Conversely, the administration of strychnine into the DRN of animals exposed to an aversive environment, such as the open field and elevated plus-maze, did not significantly influence anxiety. Similarly, McCool and Chappel (2007) have previously reported that the anxiolytic-like effects of the intra-amygdala administration of strychnine and taurine in the elevated plus-maze was dependent upon the basal anxiety state of the animals.

In addition, the intra-DRN administration of strychnine induced a suppression of locomotor activity in animals administered aCSF into the lateral ventricle. However, the strychnine-mediated suppression of locomotor activity was absent in animals administered ethanol into the lateral ventricle. The mechanism which mediated the unveiling of this locomotor effect is unknown but may be related to recovery of the animals from the surgical procedure. As discussed in *section 4.3.4.,* it may be that the anxiogenic and inhibitory properties of strychnine may be dissociable. Furthermore, these distinct effects may be mediated by distinct populations of serotonergic neurons within the DRN. An additional aim of the project was to investigate the potential interaction between the strychnine-

sensitive glycine receptors and the anxiolytic properties of ethanol. The data generated from the current study do not support such an interaction. However, this may have been due to factors related to the experimental design which will be discussed.

McDermott and Kelly (2008) reported that the Sprague-Dawley rat was significantly more anxious and less active, as measured in the elevated plus-maze and open field respectively, when compared to the Lister Hooded rat. Therefore, the strain of rat used in the present study may be inherently sensitive to anxiogenic stimuli. In agreement, previous studies in the laboratory have reported a low number of open arm entries, ratio open: total arm entires and time spent in the open runways in rats acutely exposed to the elevated plus-maze (Storey *et al*. 2006). However, Stewart *et al*. (1993) reported that the alcohol preferring P line of rats had a higher level of anxiety in the elevated plus-maze and Geller-Seifter conflict task than the NP line. In agreement, similar results were reported for the Sardinian alcohol preferring line when compared to the non-preferring strain (Colombo *et al*, 1995). Nevertheless, there may still be merit in investigating the potential interaction between the strychnine-sensitive glycine receptors and the anxioyltic properties of ethanol in an alcohol preferring strain of rat. As Colombo *et al*. (1995) reported that alcohol preferring strains are more sensitive to the stimulant effects of ethanol.

A comparison of all control groups can be seen in *table 5.1.* The number of enclosed arm entries in the elevated plus-maze appears to be relatively consistant in the control groups when compared between the systemic and centrally administered animals. Therefore, this would suggest that the route of

Experiment	Treatment	Number of Open Arm Entries	Open Arm Entries (% of Total)	Time in the Open Arms (% of Total)	Number of Closed Arm Entries
3.2	I.P. Saline (*n=10*)	8.30	18.79	9.72	36.10
3.3	Intra-DRN aCSF (*n=8*)	2.75	10.91	2.82	21.38
3.4	Intra-DRN aCSF (*n=4*)	2.75	9.43	3.81	22.00
4.1	I.P. Saline (*n=8*)	8.63	23.53	8.63	29.25
4.2	ICV aCSF (*n=4*)	2.75	6.98	2.58	33.25
4.3	ICV aCSF and Intra-DRN aCSF (*n=8*)	3.39	9.65	3.08	32.50
4.4	Intra-DRN aCSF (*n=8*)	3.25	13.34	3.43	22.38

Table 5.1. – Comparison of the control groups associated with each experiment performed in the elevated plus-maze. The table demonstrates the performance of the control groups (either the administration of saline delivered via and i.p. administration of saline or the central administration of aCSF) in the elevated plus-maze over the course of 15 min. The number of closed arm entries, which is considered an approximate measure of locomotor activity, is relatively consistant between the groups. However, the number and percentage of the total entries made into the open runways and the percentage of the trial time spent in the open runways varies between the control groups. Of particular note is the decrease in all three measures of anxiety-like behaviour in the elevated plus-maze in animals administered the experimental solution centrally when compared with animals which are administered the saline vehicle systemically.

administration had very little effect upon the locomotor activity of the animals in the elevated plus-maze. However, a comparison of the three indices of anxiety-like behaviour in the elevated plus-maze (the number and percentage of entries into the open runways and the percentage of the trial time spent in the open arms) suggests that animals which have been administered the experimental solution centrally show a higher basal level of anxiety in elevated plus-maze as demonstrated by a decrease in the number and percentage of entries into the open runways and the percentage of the trial time spent in the open runways when compared to animals administered the vehicle solution systemically.

Therefore, the sensitivity of the elevated plus-maze to measure anxiogenic-like effects may be compromised by the high basal anxiety-state of the animals. This corroborates the hypothesis that the high-basal anxiety state of animals administered the vehicle solution centrally may have resulted in a ceiling effect which may haveconfounded the experimental findings. The inclusion of additional behavioural tests, which have shown sensitivity to treatments which modulate the serotonergic system, such as the Vogel or Geller-Seifter conflict tasks may have been recruited to the study. However, as was observed in the open field, the high basal anxiety state of the animals may be a confounding factor in the investigation of the strychnine-sensitive glycine receptors independent of the behavioural test used to access the emotional state of the animals.

The high basal state of anxiety observed in the open field and elevated plus-maze may be a consequence of the microinjection procedure. Acute restraint stress (15 mins) immediately prior to testing, which was necessary in the present study to administer substances into the DRN, has been reported to reduce the percentage of the total entries into and the time spent exploring the open runways of the elevated plus-maze (Gameiro *et al*, 2006). Furthermore, Cheeta *et al*. (2001) reported that single and groups housed animals were found to have significant differences in the social interaction test. Therefore, the necessity to individually house animals administered strychnine into the DRN may have been a contributing factor.

Weis and Heller (1969) suggested that diameter of the cannulae used to administer substances into discreet nuclei must be minimal to account for damage to the target site. In the present study, attempts were made to minimise the effects of mechanical damage to the DRN by using 36 ga. microinjection

needles. However, the acute mechanical damage to the target site may have contributed to the high basal state of anxiety observed. In particular, the mechanical damage to the target site may have resulted in a local release of serotonin prior to the administration of strychnine.

Greenshaw (1986) has previously suggested that the experimental outcomes in response to the administration of substances centrally are influenced by factors associated with the composition of the administered solution. In this respect, the concentration and the volume of the solution administered must be carefully considered. The volume of the solution must be minimal, as larger volumes can damage the target site mechanically by displacement. Therefore, in the present study the volume administered into the DRN was 500 nl, comparable to previous studies, and therefore unlikely to cause significant damage to the target site. However, the use of small injection volumes can damage the target site through osmotic pressures as this necessitates the use of higher concentrations of the substance. Unfortunately, this was not controlled for in the present study. Ideally, an inactive isomer of the experimental substance should be administered as an additional control group. A further consideration is the pH of the solution administered (Greenshaw, 1986), which was controlled for in the present study by the artificial cerebrospinal fluid (aCSF). However, the state-specific nature of the data generated in the present study would suggest that the experimental outcomes were pharmacological in nature and not due to non-specific effects.

In addition, the lipid solubility of the solution is a consideration (Greenshaw, 1986). Highly lipophilic compounds diffuse rapidly in the brain and therefore may evoke behavioural responses at sites distal to the target nuclei. In the present study, this may have influenced the central administration of ethanol

particularly within the DRN. The findings of the present study suggest that the ICV administration of ethanol may be an appropriate route of administration in the study of the anxiolytic properties of ethanol. The central administration of ethanol has appeal, as the concentration of ethanol within the CNS can be controlled. However, the ICV administration of ethanol may not mimic the distribution of ethanol within the CNS following the consumption of ethanol and therefore may have limited translational validity.

Previous studies have investigated the effects of substances upon the serotonergic neurons in the DRN *in vivo* using the intracerebral microinjection technique (Higgins *et al*, 1988; Higgins and Elliot, 1991; Hillegaart, 1990; Hogg *et al*, 1994). However, an alternative approach to administer substances into the DRN was considered in the present study. In a process referred to as reverse-microdialysis, substances can be administered centrally via an implanted microdialysis probe through the inclusion of the compound in the ringer solution which perfuses the probe (Molander and Soderpalm, 2005; Molander *et al*, 2005; Chau *et al*, 2010). However, the administration of substances via microinjection was favoured in the present study as the microdialysis probe must be implanted directly into the target site, which can cause damage to the tissue proximal to the probe (Fumero *et al*, 1994). In addition, the implantation of a permanent microdialysis probe stimulates glial growth in the surrounding tissue and can limit the diffusion of substances across the membrane (Imperato and Di Chiara, 1984; Benveniste and Diemer, 1987). Therefore, the viability of administering substances via a permanent microdialysis probe is limited to 72 hr post-surgery. This would reduce the duration of the recovery period available to the animals and may exacerbate the high basal state of anxiety observed in the present study.

At the neuronal level, these state-dependant effects observed in the present study may due to the mechanism by which strychnine mediates the inhibition of the strychnine-sensitive glycine receptor. Previously, O'Connor *et al*. (1996) and Grudzinska *et al*. (2005) identified the residues which the mediate the binding of the antagonist to the glycine receptor. The binding site is localised at a distinct, but overlapping site to the endogenous agonist binding site. Therefore, the lack of measurable behavioural outcomes in response to the administration of strychnine may be due to the concentration of the endogenous agonist in the anxious state. Previous studies, have shown that both the open field and elevated plus-maze are anxiogenic stimuli (Pellow *et al*, 1985; Walsh and Cummings, 1976) and are therefore anticipated to facilitate serotonergic transmission originating from the serotonergic neurons of the DRN (Langen *et al*, 2002). A facilitation of serotonergic transmission from the DRN would be mediated by an increase in neuronal excitation. Therefore, it is inferred that the activation of the strychnine-sensitive glycine receptor upon exposure to the apparatus may be low or negligible. As described by Pellow *et al*. (1985), the elevated plus-maze was designed as an assay for the development and identification of novel anxiolytic compounds and therefore may be more sensitive to manipulations which alleviate anxiety as opposed to the facilitation of anxiety.

In this regard it would of value to investigate the effects of activation of the strychnine-sensitive glycine receptors in the DRN on anxiety-like behaviours in future studies. A study was performed in the elevated plus-maze to investigate the effects of the administration of glycine into the DRN. However, the endogenous agonists of the glycine receptor lack pharmacological specificity (*section 1.5.4.*), which may confound the interpretation of the data. In addition,

the extracellular concentrations of the endogenous agonist are regulated by membrane-bound transporters (*section 1.5.3.*). Therefore, this may necessitate the use of high concentrations to be administered and may results in non-specific effects due to osmotic pressure. However, an alternative may be to administer GlyT1, GlyT2 or TauT inhibitors into the DRN to increase the local concentration of the endogenous agonist. Given the aversive components of the microinjection protocol and the nature of the behavioural tests of anxiety, it may advantageous to investigate a putative anxiolytic response as opposed to an anxiogenic response.

Furthermore, it would be of significant value to directly measure the effects of strychnine and ethanol administration into the DRN on serotonin overflow at a terminal domain of the serotonergic neurons. Previous studies have made inference to the neuronal excitation of the serotonergic neurons originating from the DRN by investigating serotonin overflow in the vmPFC (Storey *et al*, 2006; Bland *et al*, 2003; Langen *et al*, 2002). Such an approach could be used in conjucture with the microinjection procedure; however, a more refined approach may be to implant a microdialysis probe into the DRN in addition to the vmPFC. Despite the disadvantages discussed above, this may mitigate the effects of the aversive components of the microinjection procedure, such as restraint, as strychnine could be infused in the ringer solution (Molander and Soderpalm, 2005; Molander *et al*, 2005; Chau *et al*, 2010). An additional benefit to such an experimental approach would be that further information could be garnered in relation to the dose-dependent effects on the magnitude and duration of action of both ethanol and strychnine acting upon the strychnine-sensitive glycine receptors of the DRN. This may improve the validity of the experimental design as the both the concentration of both ethanol and strychnine and the duration of

the trial time spent in the appropriate behavioural tests could be modified appropriately.

In conclusion, the present study has demonstrated that the recently identified population of strychnine-sensitive glycine receptors expressed in the DRN play a role in the mediation of anxiety-like behaviours. Due to the nature of the techniques used in the present study, future studies should aim to investigate the neurochemical response evoked by the intra-DRN administration of strychnine. Of particular interest, would be to dissociate the GABAergic-mediated behaviours from the strychnine-sensitive glycine receptor-mediated behaviours as the $GABA_A$ receptors and glycine receptors occupy distinct sites (i.e. synaptic vs. extrasynaptic) on the neuronal membrane and may therefore play functionally distinct roles in the mediation of anxiety.

6. References

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